Conserved Genetic Modules Controlling Lateral Organ Development: Polycomb Repressive Complex 2 and ASYMMETRIC LEAVES1 Homologs in the Lower Eudicot Aquilegia (Columbine).

Conserved genetic modules controlling lateral organ development: Polycomb Repressive Complex 2 and ASYMMETRIC LEAVES1 homologs in the lower eudicot Aquilegia (columbine).

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Abstract

Development in multicellular organisms relies on establishing and maintaining gene expression profiles that give cells identity. Transcription factors establish gene expression profiles by integrating positional, temporal, and environmental cues to regulate genes essential for a cell's identity. These signals are often short lived while the differentiated state may persist for a long time. Epigenetic factors maintain these gene expression profiles by making heritable chemical alterations to target gene chromatin to stabilize transcriptional patterns. Here we explore the evolution and function of an epigenetic regulator, the Polycomb Repressive Complex 2 (PRC2), and a transcription factor, *ASYMMETRIC LEAVES* 1 (*AS1*), in the lower eudicot *Aquilegia*.

PRC2 is an important and deeply conserved epigenetic regulator, which is critical to many plant developmental processes, including the regulation of major developmental transitions and lateral organ development. We find that *Aquilegia* has a relatively simple complement of PRC2 genes that are expressed throughout development. Contrary to findings in other plant species, two members of the *Aquilegia* PRC2, *AqSWN* and *AqCLF,* are not imprinted in *Aquilegia* endosperm. Using virus-induced gene silencing (VIGS), we determined that *Aquilegia* PRC2 regulates aspects of lateral organ development, including branching within the leaf and lamina expansion,

along with caroteinoid production in floral organs. PRC2 targeting of several floral MADS box genes may be conserved in *Aquilegia*, but other known targets such as the class I KNOX gene are not.

AS1 is a transcription factor that plays a conserved role in controlling differentiation and polarity of lateral organs. In species with simple leaves, *AS1* promotes cell determination by suppressing the expression of the class I KNOX genes in leaf primordia and regulates abaxial-adaxial polarity in the developing leaf. However, in species with compound leaves, KNOX genes and *AS1* often work together to control leaflet initiation and arrangement. In *Aquilegia, AqAS1* appears to primarily contribute to proper regulation of class I KNOX genes with a more minor role in leaflet polarity and positioning. Most interestingly, these combined datasets suggest that contrary to the widely held model, class I KNOX genes are neither necessary nor sufficient for leaf complexity in *Aquilegia*.

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Glossary of Terms

Abaxial – Ventral or lower surface of the leaf.

Adaxial – Dorsal or upper surface of the leaf.

Anthesis – The stage in flower development when the stamens mature.

Anthocyanin – Water-soluble vacuolar pigments that are red, purple, or blue depending on pH.

Carotenoid – Fat-soluble yellow or orange pigments found in chloroplasts.

Carpel – Structure that encloses the ovules in flowering plants.

Chromatin Remodeling Factor – Proteins that control gene expression by altering chromatin architecture to promote or restrict access to the DNA.

Compound Leaf – A leaf where the lamina is subdivided into several leaflets that are arranged around a central rachis.

Endosperm - A nutritive tissue found in angiosperm seeds that contains two maternal and one paternal genomic complements.

Epigenetics – Heritable changes in gene expression that are not caused by changes in gene sequence.

Eudicot – A monophyletic group of flowering plants that includes most dicots, or plants whose embryos have two leaves or cotyledons.

Gametophyte – The haploid multicellular generation in plants which produces gametes by mitosis.

Heteroblasty – Several forms or type of leaf.

Histone – Proteins that associate with DNA and condense it into chromatin.

Homolog – A gene related to a second gene by decent from a common DNA sequence.

Imprinting – Certain genes that are expressed in a parent of origin specific manner.

Inflorescence – A meristem that produces flowers in the axils of its leaves.

Lateral Organ – A terminally differentiated organ of the plant. They include leaves and floral organs.

Leaf – Typically the main photosynthetic lateral organ of most plants. They consist of a flat lamina, a petiole, and a leaf base.

Leaflet – A subdivision of the lamina in compound leaves.

Meristem – A population of pluripotent stem cells in plants.

Micropyle – The region of the ovule where the pollen tube enters.

Monocot – A monophyletic group of flowering plants whose embryos have one leaf or cotyledon.

Non-Peltate – Palmately compound leaves that lack a leaflet in the adaxial most position.

Ortholog – A gene related to a second gene in another species by decent from a common DNA sequence by speciation.

Palmate – A compound leaf where the leaflets are clustered at the tip of the rachis.

Paralog – A gene related to another gene by duplication within a genome.

Peltate – Palmately compound leaves where the leaflets are arranged all around the tip of the rachis.

Perianth – The sterile organs in a flower, typically sepals and petals.

Petal Limb – The flattened lamina or expanded tip of the petal.

Petal Spur – A tubular projection from the petal that often contains nectar.

Petal – Sterile second whorl organs in the flower.

Petiole – The stalk that attaches the lamina of the leaf to the stem.

Petiolule – The stalk of a leaflet in a compound leaf.

Phyllotaxy – The arrangement of the leaves around the stem.

Pinnate – A compound leaf where the leaflets are arranged all around the rachis.

Pluripotent – An undifferentiated cell that has the potential to differentiate into any cell type in a body of an organism.

Rachis – The main axis of a compound leaf (see petiole).

Sepal – Sterile first whorl organs in the flower.

Shoot Apical Meristem – A population of pluripotent stem cells located at the tips of growing shoots.

Simple Leaf – A leaf with an undivided lamina.

Sporophyte – The diploid multicellular generation in plants which produces spores by meiosis.

Stamen – The male reproductive part of the plant. Typically consists of a pollen bearing anther attached to the plant by a stalk called the filament.

Staminodia – A novel sterile organ in the *Aquilegia* flower that are located between the stamens and the carpels.

Stomata – A pore in the epidermis of the plant that allows for gas exchange.

Subfunctionalization – A type of functional divergence after a gene duplication event in which each paralog retains a subset of the ancestral function of the gene.

Transcription Factor – A protein that binds to DNA near genes and controls the expression of those genes.

Vernalization – An extended cold treatment that promotes the transition to flowering in some plant species.

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Chapter 1:

Introduction

Plants and animals diverged approximately 1.6 billion years ago, before the evolution of multicellular organisms (Reviewed in: Meyerowitz 2002). Thus, multicellularity is thought to have evolved independently in these two groups. However, in both lineages development relies on establishing and maintaining gene expression profiles that give cells identity. Gene expression profiles are created when a cell integrates positional, temporal, and environmental cues to activate or silence genes essential for their identity. These signals are then interpreted by different factors in the cell, including transcription factors and epigenetic regulators that ultimately control multicellular development. In this study we explore the evolution and function of a transcription factor, *ASYMMETRIC LEAVES* 1 (*AS1*), and an epigenetic regulator, the Polycomb Repressive Complex 2 (PRC2), in the lower eudicot *Aquilegia*.

One way that cells respond to developmental signals is though transcription factors. Transcription factors are proteins that bind to DNA near genes. They then either activate the transcription of the target gene by recruiting the transcriptional machinery or repress its transcription through a number of mechanisms. Often during development, so called ‘master regulator’ transcription factors are activated by developmental signals and alter the expression of hundreds of genes, thus specifying a particular cell fate (Reviewed in: Oestreich and Weinmann 2012). However, the signals that activate these transcription factors are often short lived while the differentiated state may persist for a long time.

Gene expression can be maintained in a heritable fashion via epigenetic regulation. Epigenetics is defined as heritable changes in gene expression that are not caused by changes in gene sequence (Reviewed in: Holliday 1994; Russo et al. 1996; Feil 2008). These changes function by making heritable chemical alterations to target gene chromatin and thereby stabilize transcriptional patterns. Such chemical alterations can range from DNA methylation to covalent modifications of histone tails (Reviewed in: Feil 2008). Although DNA methylation always results in gene repression, histone modifications are interpreted by the cell as either repressing or promoting transcription of the adjacent loci depending on the type of modification (Reviewed in: Jaenisch and Bird 2003). In some cases, as with histone acetylation, these modifications function by altering the affinity of histone tails for DNA thus making the DNA more or less accessible to the transcriptional machinery (Reviewed in: Kuo and Allis 1998). In other cases, the effect of the particular histone modification expression has no obvious chemical explanation. The 'histone code' hypothesis theorizes that covalent modifications to the histone tails recruit other chromatin regulatory proteins which in turn affect transcription (Jenuwein and Allis 2001). In this introductory chapter, I will consider both of these regulatory mechanisms in the context of plant development, specifically the production of complex lateral organs.

1.1: Protein Effectors of Epigenetic Modification and their Functions in Plants

Many proteins involved in epigenetic maintenance of gene expression are highly conserved between plants and animals and appear to function in a remarkably similar

components including Polycomb, Polyhomeotic, Posterior Sex Combs, and dRing (Reviewed in: Schuettengruber et al. 2007). This complex binds to the H3K27 trimethylation deposited by PRC2 and stably represses gene expression (Fig. 1.1A).

Polycomb Repressive Complex 2 (PRC2) is most likely homologous between plants and animals, however there are some differences in plants (Fig. 1.1B) (Reviewed in: Schuettengruber et al. 2007; Whitcomb et al. 2007). The *E(z)* lineage in plants underwent an ancient duplication such that most angiosperms have at least two paralogs, known as *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*) (Spillane et al. 2007). *Suppressor of Zeste 12 (Su(z)12)* and *Extra Sex Combs (ESC)* are known in plants as *EMBRYONIC FLOWER 2* (*EMF2*) and *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), respectively (Fig. 1.1B) (Reviewed in: Pien and Grossniklaus 2007). The core PRC2 proteins have been duplicated in many plant species including *A. thaliana* which allows these species to form several PRC2s with distinct developmental functions (Fig. 1.1B) (Reviewed in: Whitcomb et al. 2007).

The PRC1 is not thought to be homologous between plants and animals, but there appears to be a complex that plays a functionally analogous role. This complex appears to include RING finger proteins, similar to the animal PRC1 complex; LHP1, a plant homolog of the animal protein HP1 that is not found in the animal PRC1 complex; and EMF1, a plant specific protein (Fig. 1.1B) (Calonje et al. 2008; Xu and Shen 2008; Exner et al. 2009; Bratzel et al. 2010; Beh et al. 2012).

manner (Reviewed in: Pien and Grossniklaus 2007; Whitcomb et al. 2007; Köhler and Hennig 2010). One key example is the Polycomb Group (PcG). These proteins were first discovered in *Drosophila melanogaster* as repressors of the HOX genes, a class of transcription factors that regulate the creation of a segmented body plan and the specification of cell fate in animals (Lewis 1978). PcG proteins do not create the initial Hox gene expression pattern but instead are required to maintain appropriate silencing after the initial regulatory signals disappear (Reviewed in: Schuettengruber et al. 2007). Since their discovery in *Drosophila*, homologs of the PcG have been identified in a number of diverse taxa from across the metazoa, fungi, and plants (Reviewed in: Sawarkar and Paro 2010). The PcG proteins have also been shown to modulate gene expression in a wide array of important developmental processes including pluripotency, cell cycle regulation, imprinting, response to environmental signals, and cellular differentiation (Reviewed in: Sawarkar and Paro 2010).

In animals, the PcG proteins form several complexes with distinct functions (Fig. 1.1A). Two such complexes, known as Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2), function in tandem (Schuettengruber et al. 2007). PRC2 contains four core proteins: the histone methyltransferase *Enhancer of Zeste* (*E(z))* and three other proteins thought to enhance PRC2 binding to nucleosome (Nekrasov et al. 2005), *Suppressor of Zeste 12 (Su(z)12), Extra Sex Combs (ESC)*, and *Multi-Copy Suppressor of IRA 1 (MSI1*) (Fig. 1.1A) (Reviewed in: Pien and Grossniklaus 2007). The PRC2 complex represses gene expression by trimethylating lysine 27 of histone H3 (H3K27) (Schubert et al. 2006). The PRC1 contains several core

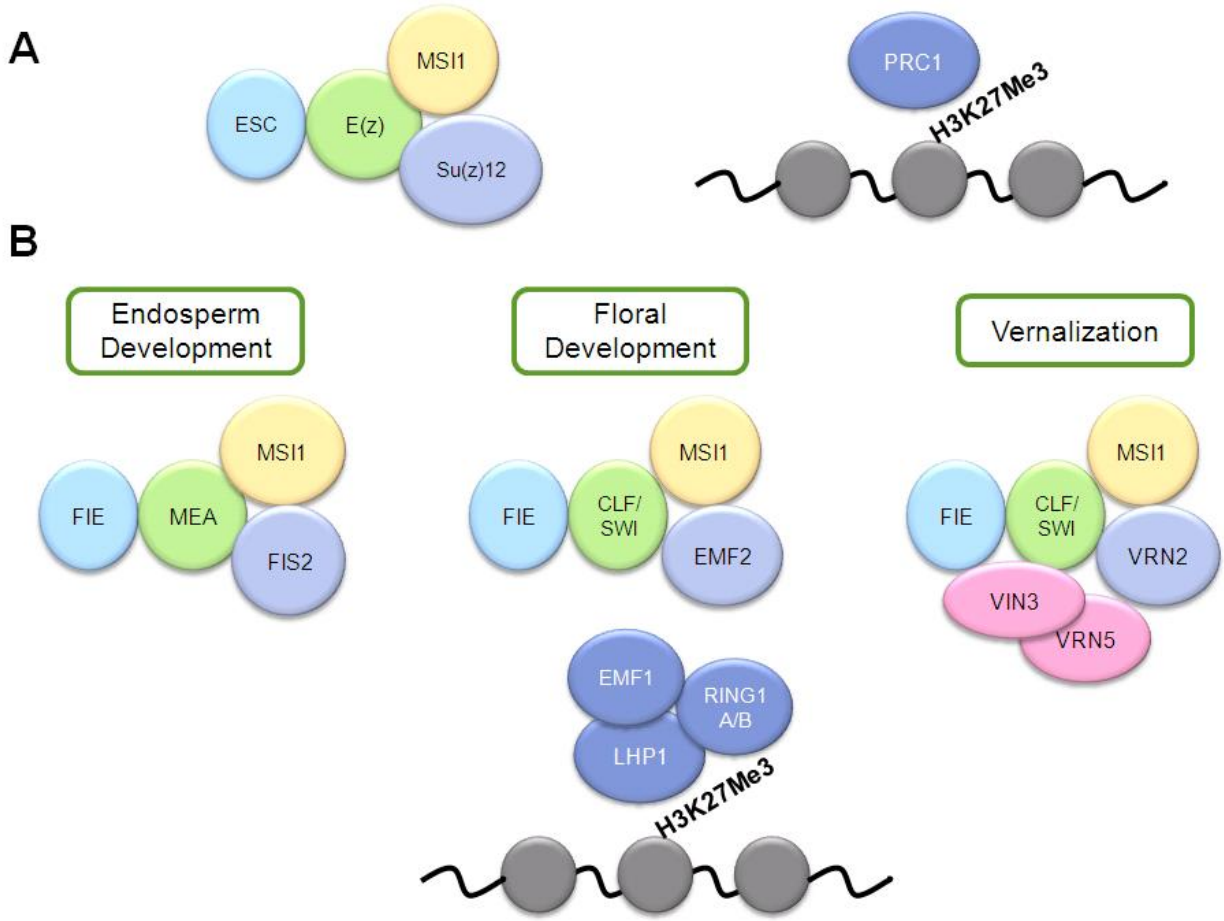


Figure 1.1: Summary of Polycomb function in animals and plants. **A.** The core components of the Polycomb Repressive Complex 2 (PRC2) in *Drosophila*; MSI1, ESC, Su(z)12, and E(z)**.** This complex suppresses transcription of different loci by trimethylating lysine 27 on histone H3 (H3K27Me3). In *Drosophila,* the Polycomb Repressive Complex 1 (PRC1) then binds to the H3K27Me3 and stably represses gene expression (Reviewed in: Schuettengruber et al. 2007). **B.** Su(z)12 and E(z) have several homologues in *A. thaliana* that are known to form different complexes involved in various aspects of plant development. *FIS2* and *MEA* are expressed in the seed and required for proper endosperm development. The paralogs *CLF* and *SWI* act redundantly along with *EMF2* to repress early flowering and promote proper expression of at least one floral homeotic gene (Goodrich et al. 1997). *CLF* and *SWN* also interact with *VRN2* to repress the floral repressor *FLC* during vernalization (Chanvivattana et al. 2004).

Figure 1.1 Continued: *VRN5* and *VIN3* have been found to bind to this complex during vernalization (De Lucia et al. 2008). *MSI1*, and *FIE*, the Esc homolog, have been found in all Polycomb complexes characterized to date (Reviewed in: Sung and Amasino 2005). Recently a complex was identified in *A. thaliana* that consists of *LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), EMBRYONI FLOWER 1 (EMF1), RING1a,* and *RING1b* and was shown to bind H3K27Me3 via the chromo-domain in LHP1 (Xu and Shen 2008). This complex is hypothesized to function similarly to PRC1 in *Drosophila.*

Studies have shown that PRC2 is involved in developmental transitions in a number of plant species beginning very early in development and continuing throughout the plant life cycle (Goodrich et al. 1997; Gendall et al. 2001; Yoshida et al. 2001; Kohler et al. 2003b). In the plant model system, *Arabidopsis thaliana*, the genes *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) a Su(z)12 homolog, *FIE*, *MSI1*, and *MEDEA (MEA),* an E(z) homolog, are involved in endosperm development (Luo et al. 1999; Ohad et al. 1999; Spillane et al. 2007). Endosperm is a nutritive tissue found in angiosperm seeds that contains two maternal and one paternal genomic complements (Baroux et al. 2002). Mutations in these genes cause aberrant development of the endosperm of fertilized seeds resulting in embryo abortion around the heart stage and precocious endosperm development in unfertilized gametophytes (Ohad et al. 1996; Luo et al. 1999; Guitton et al. 2004). Interestingly, PRC2 plays a role in differential imprinting of loci in the maternal and paternal genomes of developing embryos and endosperm. Furthermore, members of the PRC2 complex itself have been found to be imprinted in

A. thaliana and several grasses (Kinoshita et al. 1999; Springer et al. 2002; Guitton et al. 2004; Luo et al. 2009).

In the cases of *MEA*, *FIS2*, and *FIE*, the loci are imprinted in the endosperm such that the maternal copies are expressed while the paternal copies are silenced (Guitton et al. 2004). During vegetative growth, both *FIS2* and *MEA* are silenced epigenetically, *FIS2* by DNA methylation and *MEA* by H3K27 trimethylation added by the PRC2 complex (Katz et al. 2004; Jullien et al. 2006a; Jullien et al. 2006b). These genes are activated only in the central cell of the female gametophyte by a DNA repair complex that has been shown to remove DNA methylation on *FIS2* (Jullien et al. 2006b). Activation of *MEA* is different from *FIS2* and is not completely understood, but involves removal of the H3K27me3 and also removal of DNA methylation (Jullien et al. 2006a; Jullien et al. 2006b). *DEMETER,* a critical component of the DNA repair complex that activates *FIS2* and *MEA,* is not expressed in the male gametophyte, which may explain why *MEA* and *FIS2* are not activated in the paternal genome (Choi et al. 2002). While *FIE* is expressed during vegetative growth during embryogenesis, *FIE* is expressed from the maternal allele alone early in endosperm development, but the paternal allele becomes activated at a late stage (Ohad et al. 1999).

Although few targets of the FIS PRC2 complex have been identified in *A. thaliana*, it is thought that they help control cell proliferation in and nutrient allocation to the endosperm (Reviewed in: Hsieh et al. 2003). It has also been shown that the FIS PRC2 complex is responsible for regulating the expression of several genes that are

themselves imprinted (Reviewed in: Rodrigues et al. 2010). One such gene, a type 1 MADS box gene called *PHERES1* (*PHE1*), is expressed only from the paternal allele in the endosperm while the maternal allele of *PHE1* is silenced by the FIS complex (Kohler et al. 2005). Downregulation of *PHE1* has been shown to restore embryo viability in a *mea* background, thus controlling gene dosage of *PHE1* may be an important function of the PRC2 complex in endosperm development (Kohler et al. 2003a) .

Recent work has demonstrated that PRC2 complex members are also imprinted in other species and suggests that this may be a common theme in endosperm development. A maize E(z)-like gene (*Mez1*), maize *ZmFIE1*, and rice *OsFIE1* are imprinted in the endosperm (Springer et al. 2002; Haun et al. 2007; Luo et al. 2009). However, mutations in *OsFIE* caused by T-DNA insertions did not induce over-proliferation of the endosperm, suggesting that these genes are not involved in controlling endosperm proliferation (Luo et al. 2009). Additionally, *Mez1, ZmFIE1,* and *OsFIE1,* as well as *MEA* and *FIS2*, all arose from recent duplication events in their respective lineages (Reviewed in: Rodrigues et al. 2010). One possibility is that the genetic redundancy resulting from these duplication events freed these genes to evolve new or subfunctionalized roles in endosperm development.

During vegetative development, the main components of this complex in *A. thaliana* appear to be *FIE*, *MSI1*, *CLF* or *SWN*, and *EMF2* (Chanvivattana et al. 2004). Weak silencing of *EMF2* results in curled leaves and ectopic expression of *AG* similar to *CLF* mutants (Chanvivattana et al. 2004). However, strong *EMF2* mutants completely skip

vegetative development and instead produce a small inflorescence immediately upon germination (Yoshida et al. 2001). This phenotype is thought to be due in part to de-repression of genes important in floral meristem and floral organ identity during embryogenesis and suggests that the PRC2 complex may play an early role in repressing reproductive development (Reviewed in: Hsieh et al. 2003). Interestingly, T-DNA insertions in rice *OsEMF2b* flowered early in long day conditions although, unlike in *A. thaliana,* some vegetative growth was observed (Luo et al. 2009). *OsEMF2b* mutants also displayed defects in floral organ morphology (Luo et al. 2009). It is possible that the role of the PRC2 complex repressing early flowering and regulating floral development may be at least partially conserved.

The role of PRC2 repression in developmental transitions in *A. thaliana* is perhaps best understood during the transition to flowering. Precise control over the transition to flowering is essential to a plant's reproductive success. Thus, flowering time is heavily regulated in many plants by both internal signaling pathways (the autonomous and gibberellin pathways) and environmental cues such as day length, ambient temperature, and exposure to long periods of cold, called vernalization (Reviewed in: Sung and Amasino 2005). In certain ecotypes of *A. thaliana*, known as winter annuals, epigenetic regulation plays an important role in mediating the response to vernalization (Reviewed in: Hsieh et al. 2003; Sung and Amasino 2005). Before vernalization a MADS box gene called *FLOWERING LOCUS C (FLC)* prevents flowering by repressing loci that are critical to inflorescence and floral meristem identity including *FLOWERING LOCUS T* (*FT*) and *SUPRESSOR OF CONSTANS 1* (*SOC1*) (Fig. 1.2A) (Reviewed in: Hsieh et al.

2003). During vernalization, *FIE, MSI1, CLF* or *SWN*, and *VERNALIZATION 2* (*VRN2*), a Su(z)12 homolog, associate with the *FLC* locus and repress its transcription (Reviewed in: Sung and Amasino 2005). With *FLC* stably repressed, plants are able to transition to flowering in the spring.

The genetic basis of vernalization has also been studied in the monocots barley and wheat, but there seems to be little conservation between the loci involved in vernalization in these systems and those that function similarly in *A. thaliana* (Fig. 1.2B) (Reviewed in: Dennis and Peacock 2007). Thus, it is thought that the vernalization response evolved independently in these two systems. However, recent work on epigenetic regulation in grasses suggests that chromatin-based regulation may be a commonality between these two divergent pathways, despite the fact that target loci are not homologous (Oliver et al. 2009). In barley and wheat *VERNALIZATION 1* (*VRN1*) is suppressed before vernalization. Another protein, *VERNALIZATION* 2 (*VRN2*) (with no relationship to *VRN2* in *A. thaliana*) prevents flowering by repressing an *FT* homolog *Hd3a* in long day conditions (Fig. 1.2B). During vernalization *VRN1* is turned on, allowing it to repress *VRN*2 and, in conjunction with *Hd3a*, promote flowering in inductive photoperiods (Reviewed in: Trevaskis et al. 2007). Oliver et al used Chromatin Immunoprecipitation (ChIP) to analyze changes in histone modifications at the *VRN1* locus in barley in response to vernalization. They used antibodies against H3K27 trimethylation and H3K4 trimethylation, a histone modification associated with genes that are being actively transcribed (Oliver et al. 2009). Before vernalization, important regulatory regions in the barley *VRN1* locus contained high levels of H3K27

trimethylation (Oliver et al. 2009). After vernalization, H3K4 trimethylation in these regions increased and H3K27 trimethylation decreased (Oliver et al. 2009). These results are consistent with the expression pattern of *VRN1* in barley and suggest that epigenetic regulation, possibly even by the PcG, may be involved in mediating the response to vernalization in barley. The authors analyzed the histone modifications on the *VRN2* and *FT* loci, but found that the levels of H3K27 and H3K4 trimethylation did not change in response to vernalization (Oliver et al. 2009).

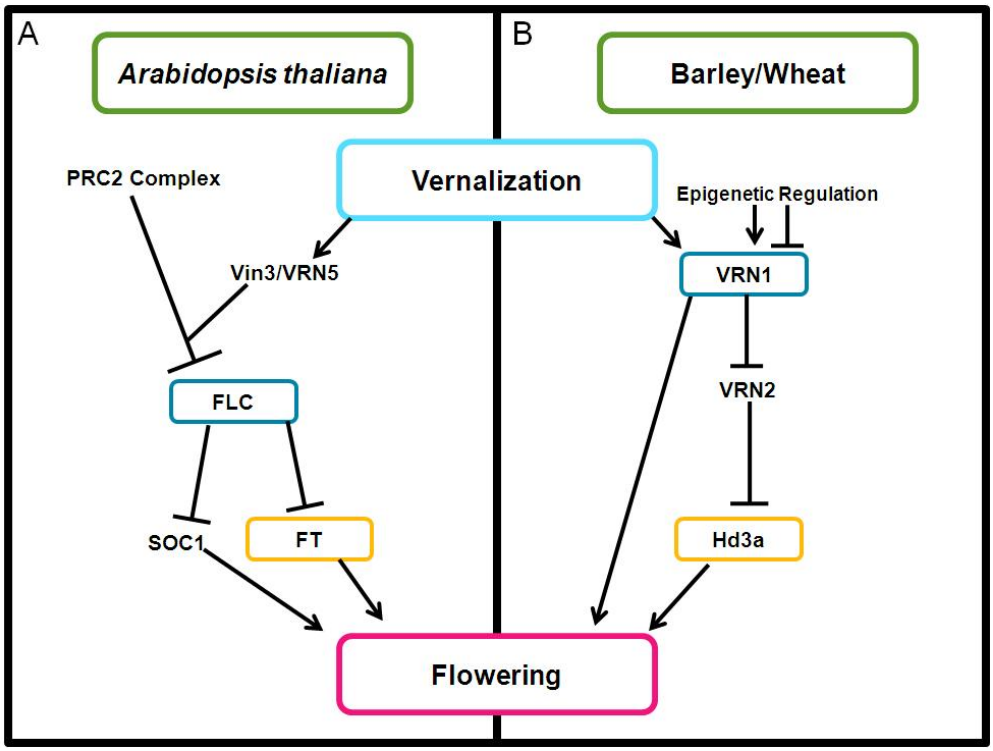


Figure 1.2: A simplified diagram of genetic responses to vernalization in *A. thaliana* and the grasses. **A.** In *A. thaliana*, *FLC* prevents flowering by suppressing floral promoting genes such as *FLOWERING LOCUS T* (*FT*) and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). *VERNALIZATION INSENSITVE 3* (*VIN3*), *VERNALZATION 5* (*VRN5*) and *VEL1* are turned on in response to vernalization (De Lucia et al. 2008). They interact with PRC2 and direct

Figure 1.2 Continued: Polycomb mediated repression of *FLC*. This allows flowering to occur in response to inductive long day photoperiods in the spring (Reviewed in: Dennis and Peacock 2007). **B.** In barley and wheat, *VERNALIZATION 1* (*VRN1*), a homolog of the *A. thaliana* floral meristem identity gene *APETALA1* (*AP1*), is suppressed before vernalization while *VERNALIZATION* 2 (*VRN2*) prevents flowering by repressing the *FT* homolog *Hd3a* in long day conditions. During vernalization, *VRN1* is turned on, allowing it to repress *VRN*2 and, in conjunction with *Hd3a*, promote flowering in inductive photoperiods (Reviewed in: Trevaskis et al. 2007). The *VRN1* locus has recently been shown to be trimethylated at H3K27 before vernalization and contain H3K4 trimethylation after vernalization (Oliver et al. 2009).

PRC2 associate with other proteins that help recruit them to specific loci in both plants and animals, (Reviewed in: Köhler and Hennig 2010; Margueron and Reinberg 2011). In *A. thaliana*, members of a plant specific group known as the VIL (VIN3-like) or VEL PHD family have been shown to associate with the PRC2 complex and seem to be required for PRC2 function during vernalization (Sung et al. 2006; Greb et al. 2007; De Lucia et al. 2008). One member of this family, *VERNALIZATION INSENSITIVE 3 (VIN3)* is silenced during normal vegetative development and is only expressed after sufficient cold exposure (at least 20 days) (Sung and Amasino 2004). The level of *VIN3* expression increases the longer the plant spends in cold temperatures (Sung and Amasino 2004). Immunoprecipitation pull down experiments with epitope tagged *VRN2* showed *VIN3* is associated with the PRC2 complex during vernalization (Figs. 1.1B) and it is thought to help mediate the interaction between this complex and the *FLC* locus (Fig. 1.2 A) (Wood et al. 2006). This is interesting because it reflects the plant's

need to measure the length of time it's been exposed to cold. The vernalization requirement in winter-annuals has evolved to ensure that the plants wait to flower until after winter. If plants responded to cold by immediately silencing *FLC*, they could be induced transition to reproductive growth after a short cold snap in the fall which could be detrimental to their offspring. Thus, the gradual induction of *VIN3* during cold exposure may part of whatever process winter-annuals use to measure the length of cold exposure. VEL PHD homologs are also induced by vernalization in wheat (Fu et al. 2007a). However it is not known if these genes associate with the wheat PRC2 or if they actually function in the floral promotion pathway.

PRC2 also plays major roles in lateral organ development in *A. thaliana*. PRC2 function was in fact first discovered in plants with the characterization of the *clf* mutant in *A. thaliana* (Goodrich et al. 1997). These mutants had severely curled leaves, smaller narrower sepals and petals, and partial homeotic transformations of sepals and petals towards carpel and stamen identity. The C class MADS box gene family member *AGAMOUS* (*AG)* and *APETALA3* (*AP3*), a B class MADS box family representative, were shown to be over-expressed in *clf* mutants. Further studies have subsequently shown that the E class MADS *SEPALLATA3* (*SEP3*) is similarly up-regulated in *clf* mutants (Lopez-Vernaza et al. 2012). This suggests that the PRC2 complex is required for stable repression of these genes which was significant because MADS box genes regulate homeotic floral organ identity in plants somewhat analogously to the way HOX genes regulate segment identity in animals (Bowman et al. 1989; Bowman et al. 1991; Goodrich et al. 1997; Foronda et al. 2009; Bowman et al. 2012).

In addition to their role in regulating MADS box genes in lateral organs, PRC2 also regulates the expression of the class I KNOX genes, a family of homeobox domain containing loci in plants that have conserved roles in promoting pluripotency and cell divisions (Bharathan et al. 2002; Wagner 2003). In *FIE* co-suppressed plants also had loss of apical dominance, fasciated stems, rolled leaves with varying degrees of serration, loss of phyllotaxy in the inflorescence, and many problems with ovary and ovule development in addition to the phenotypes already described in the *clf* mutant (Katz et al. 2004). Several class I KNOX genes, including *BREVIPEDICELLUS* (*BP*), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*), and *SHOOTMERISTEMLESS* (*STM*), were over-expressed in *FIE* silenced leaves. *STM* and *KNAT2* were also found to be over-expressed in *clf* mutants, but *BP* was not. While PRC2 functions as a complex, the differences between *clf* mutants and *FIE*-silenced plants may be due to the *CLF* paralog *SWN,* which acts redundantly in some cases (Katz et al. 2004).

Relatively little is known about the function of the PRC2 complex outside of the major angiosperm models but there is some evidence that its role in regulating developmental transitions may be quite deeply conserved. In the moss species, *Physcomitrella patens*, PRC2 may regulate promote sporophyte development (Mosquna et al. 2009; Okano et al. 2009). Deletion of the PRC2 genes *PpCLF* and *PpFIE* induces sporophyte-like development and gene expression in the gametophyte (Mosquna et al. 2009; Okano et al. 2009). Furthermore, PRC2 targeting of the class I KNOX genes may be broadly

conserved as the class I KNOX genes *MOSS KNOTTED1-LIKE 2* and *5* (*MKN2* and *MKN5*) are over-expressed in *PpFIE* mutant gametophytes (Singer and Ashton 2007; Mosquna et al. 2009). While the functions of the class I KNOX genes and other PRC2 targets like the ABC class MADS box genes are thought to be deeply conserved, at least in seed plants, comparative studies of their regulation have largely focused on upstream transcription factors (Kim et al. 2003a; Maizel et al. 2005). Whether the PRC2-targeting of these genes is similarly conserved is an open question.

1.2: Transcription Factors that Regulate Lateral Organ Development in Plants

While development largely ceases in animals after the embryonic phase, plants continue to grow and develop throughout their life cycle. The shoot apical meristem (SAM), a population of continually dividing, pluripotent stem cells located at the tips of growing shoots, provides the cellular materials for plant development. Cells located along the flanks of the SAM are recruited to form lateral organs such as leaves and floral organs. Genes that promote pluripotency must be turned off in these organs while genetic networks that shape the organ are turned on. Transcription factors play a large role in determining and shaping lateral organs. While many transcription factors including the class I KNOX genes promote pluripotency in the SAM, the R2-R3 class MYB transcription factor *ASYMMETRIC LEAVES 1* (*AS1*) promotes cell determination in the leaves and also controls aspects of leaf shape. This function appears to be broadly conserved in many angiosperms (Waites and Hudson 1995; Schneeberger et al. 1998; Sun et al. 2002; Kim et al. 2003a; McHale and Koning 2004; Tattersall et al. 2005).

One major role for *AS1* is the down-regulation of class I KNOX genes in incipient leaf primordia (Schneeberger et al. 1998; Byrne et al. 2000; McHale and Koning 2004). The KNOX genes must be turned off in developing leaf primordia to allow for proper leaf development (Jackson et al. 1994; Chuck et al. 1996; Bharathan et al. 2002). In simple-leafed taxa, *AS1* expression is absent from the SAM, where KNOX genes are strongly expressed, but *AS1* expression is detected very early in leaf initiation and retained throughout leaf development while the KNOX genes are silenced in these tissues (Waites et al. 1998; Tsiantis et al. 1999; Byrne et al. 2000; McHale and Koning 2004). The leaf phenotypes of *A. thaliana as1* mutants resemble KNOX gene over-expressing lines, including downwardly curling leaves, leaves with extra lobes, aberrant vascular patterning, and ectopic shoots on the adaxial surface of the petiole (Chuck et al. 1996; Byrne et al. 2000). Three *A. thaliana* class I KNOX genes, *BP* and *KNAT2* and *KNAT6,* are ectopically expressed in *as1* leaves (Byrne et al. 2000; Semiarti et al. 2001; Hay and Tsiantis 2009). Another of the class I KNOX genes, *STM* is not ectopically expressed in *as1* leaves, but is required to repress *AS1* expression in the meristem (Byrne et al. 2000; Ori et al. 2000). In *as1* mutants, all class I KNOX genes are initially down-regulated in incipient leaf primordia (P0), but they are reactivated later in leaf development, suggesting that while *AS1* is not required for the initial down-regulation of *BP, KNAT2* and *KNAT6,* it is required to stably repress these genes in developing leaf primordia (Byrne et al. 2000).

Mutations in *AS1* homologs in other species also cause ectopic KNOX gene expression. For instance, in Maize *rough sheath2* (*rs2*) mutant phenotypes include dwarfism due to abnormal internodal growth, aberrant vascular patterning, and disruptions at the blade-sheath boundary (Schneeberger et al. 1998; Tsiantis et al. 1999). Similar to *A. thaliana*, several maize KNOX genes are ectopically expressed in older leaves of *rs2* mutants but not in the P0, suggesting that *RS2* is required to maintain repression of KNOX genes in maize leaves (Schneeberger et al. 1998; Tsiantis et al. 1999). Another feature of AS1 homolog mutations that may be due to KNOX gene over-expression is defects in proximal-distal patterning (Schneeberger et al. 1998; Tsiantis et al. 1999; Sun et al. 2002). Wild type maize leaves consist of two portions; the proximal sheath and the distal blade. However, in *rs2* leaves portions of the blade are proximalized or transformed into sheath tissue (Schneeberger et al. 1998; Tsiantis et al. 1999). In *A. thaliana*, vascular defects in *as1* mutants also suggest proximalization of the leaves (Sun et al. 2002). *PHANTASTICA* (*PHAN),* the *AS1* homolog in *Antirrhinum* and *Nicotiana* has also been shown to regulate KNOX gene expression in these species (Waites et al. 1998; Tsiantis et al. 1999; McHale and Koning 2004). *Nicotiana phan* mutant phenotypes resemble *A. thaliana as1* phenotypes in that *nsphan* mutant leaves have disorganized patterns of cell division and ectopic primordia initiation on the adaxial surface (McHale and Koning 2004).

When targeting the KNOX loci, AS1 forms a complex with several other proteins. The LOB domain containing protein, ASYMMETRIC LEAVES 2 (AS2), interacts with AS1 and has many of the same mutant phenotypes (Semiarti et al. 2001; Lin et al. 2003; Xu

et al. 2003; Phelps-Durr et al. 2005). The AS1-AS2 complex binds directly to several KNOX gene promoters in *A. thaliana* and both AS1 and AS2 are required for these interactions (Guo et al. 2008). The AS1-AS2 complex may silence KNOX genes by recruiting the chromatin remodeling factor, HIRA, to the KNOX promoter and forming a repressive chromatin state (Phelps-Durr et al. 2005; Guo et al. 2008). This AS1-AS2-HIRA complex likely interacts with other proteins to mediate KNOX gene silencing (Borghi et al. 2007; Li et al. 2012).

Leaf polarity and laminar outgrowth are also regulated by *AS1* and *AS2* in a number of species. Leaves initiate as radially symmetric bulges on the sides of the meristem, but later acquire asymmetry along three major axes; medial-lateral, proximal-distal, and abaxial-adaxial (dorsal-ventral) (Fig. 1.3) (Reviewed in: Moon and Hake 2011). The adaxial surface is closest to the SAM and thus typically receives direct sunlight while the abaxial surface is shaded and thus contains a high stomatal density to mitigate water loss. Cells in the adaxial domain are therefore specialized for photosynthesis, while the abaxial domain is specialized for gas exchange (Reviewed in: Moon and Hake 2011); Yamaguchi et al. (2012). Juxtaposition between abaxial and adaxial identity in the leaf is also thought to be essential for laminar outgrowth (Waites and Hudson 1995) (Reviewed in: Yamaguchi et al. 2012).

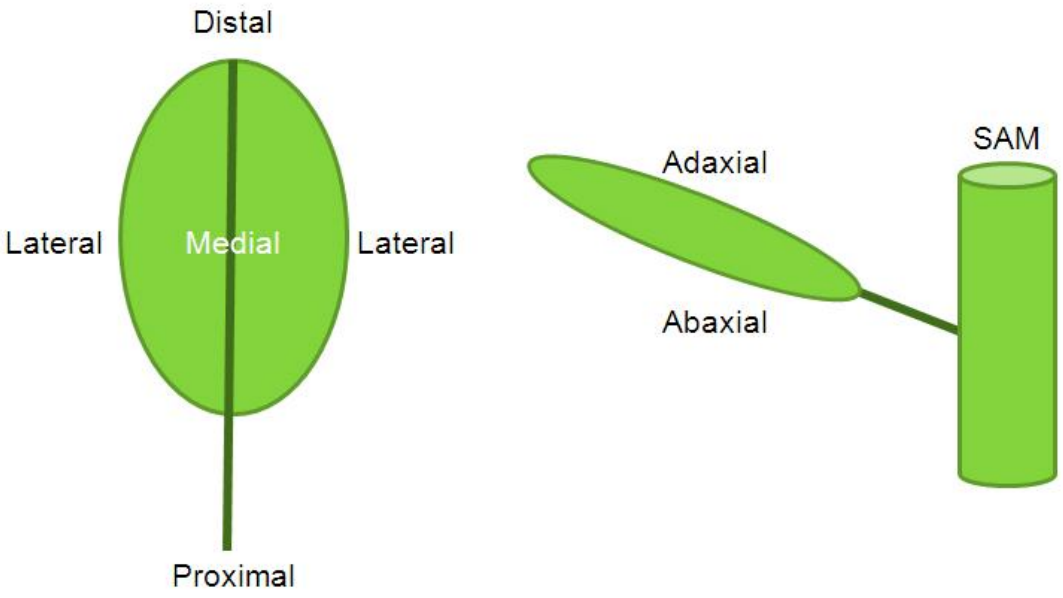


Figure 1.3: The three major axes in the leaf. These include proximal-distal, medial-lateral, and abaxial-adaxial (dorsal-ventral).

Our original understanding of the role for abaxial/adaxial identity in promoting laminar outgrowth actually comes from studies of the mutant phenotype of the *Antirrhinum AS1* homolog, *phantastica (phan),* which is largely due to alterations in abaxial-adaxial polarity (Waites and Hudson 1995; Waites et al. 1998). Weak *phan* mutant leaves are narrow or heart shaped and have patches of abaxial tissue on the adaxial surface that are surrounded by ridges of ectopic laminar outgrowth where the two identities meet, while strong mutants have needle-like leaves that are completely abaxialized (Waites and Hudson 1995). Likewise, *Nicotiana* leaves lacking *NSPHAN* expression were also needle-like and abaxialized (McHale and Koning 2004). However, the degree to which the role of *AS1* and *AS2* in abaxial identity is redundant with other pathways varies greatly across the angiosperms. Defects in abaxial-adaxial polarity were not initially

observed maize and *A. thaliana rs2* and *as1* mutants (Schneeberger et al. 1998; Byrne et al. 2000), but *AS2* is expressed only on the adaxial surface of leaf primordia, suggesting that this limited expression domain restricts AS1/AS2 function to the adaxial domain (Iwakawa et al. 2002; Xu et al. 2003). Additionally, *AS2* over-expression lines show defects in abaxial-adaxial polarity, ranging from upwardly curling, narrow leaves with patches of adaxial cell types on the abaxial surface to completely adaxialized, needle-like leaves (Iwakawa et al. 2002; Lin et al. 2003; Xu et al. 2003). Xu et al (2003) also showed that *as1* and *as2* mutants showed some abaxial-adaxial polarity defects in the *A. thaliana* Lansberg *erecta* genetic background including loss of asymmetry in the petiole and 'lotus leaves' where the petiole attached to the abaxial surface of the leaf. It is still unclear if *RS2* promotes adaxial identity in maize, but *INDETERMINATE GAMETOPHYTE 1*, a LOB domain protein with high similarity to *AS2*, has been shown to mediate aspects of abaxial-adaxial polarity suggesting that this role for the AS1-AS2 dimer may be conserved in both monocots and dicots (Evans 2007).

The variable importance of *AS1* homologs to establishing abaxial-adaxial polarity is in part due to genetic interactions between the AS1/AS2 complex and several other factors involved in leaf polarity (Lin et al. 2003; Fu et al. 2007b). Studies are still seeking to understand how the juxtaposition of abaxial and adaxial identity promotes laminar outgrowth, but recently members of the WOX family of homeodomain transcription factors were implicated in maintaining the central meristematic domain that drives this process (Nardmann et al. 2004; Vandenbussche et al. 2009; Tadege et al. 2011a;

Nakata et al. 2012). Furthermore, these WOX genes appear to interact genetically with *AS2* (Vandenbussche et al. 2009; Nakata et al. 2012).

To summarize, *AS1* homologs in *A. thaliana*, *Nicotiana,* maize, and *Antirrhinum* are important both for the negative regulation of the class I KNOX genes and for specifying adaxial identity in developing leaves. However, the relative importance of these roles varies by species resulting in the gradient of phenotypes observed (Fig. 1.4). However, these species all have simple leaves. Thus, while the functions of *AS1* are well understood simple leaf development, *AS1* has been less well studied in species with compound leaves.

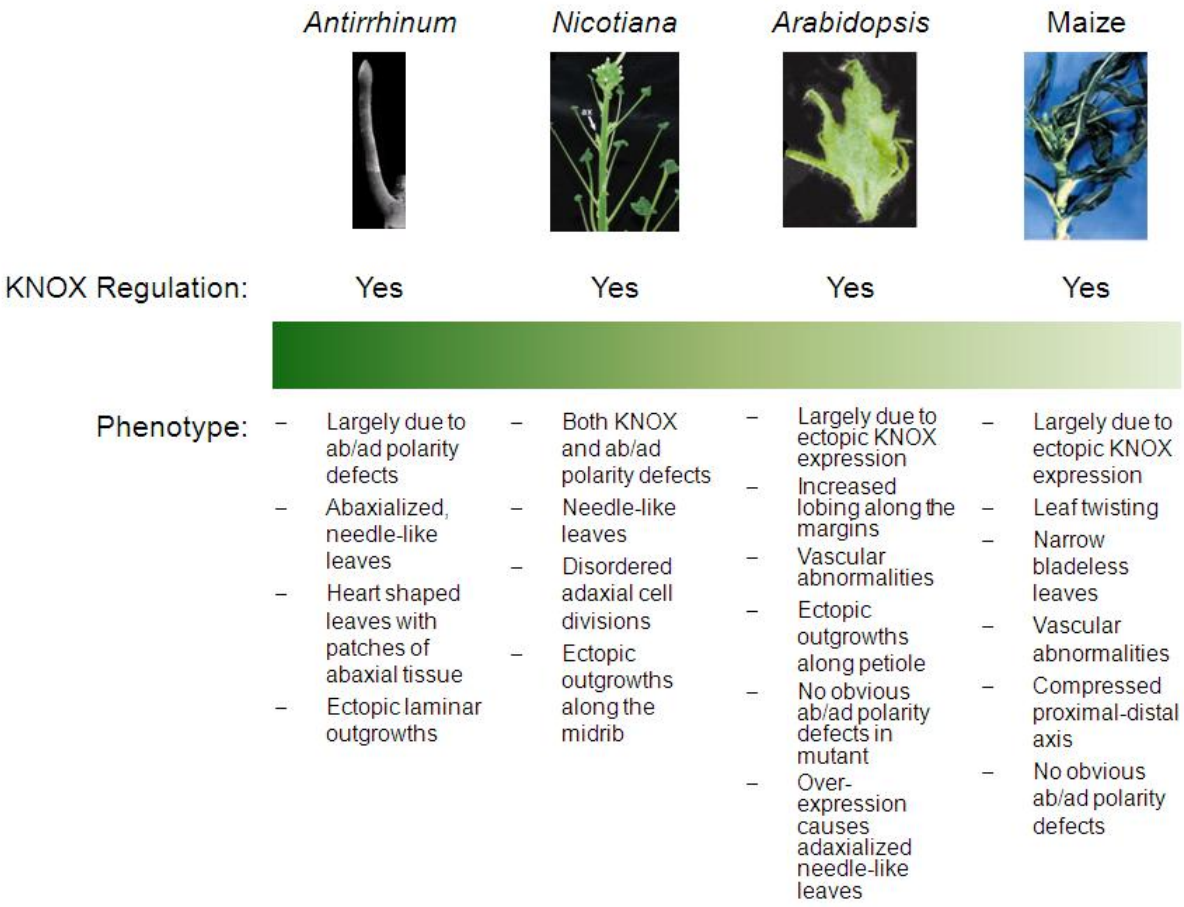


Figure 1.4: The gradient of phenotypes seen in *as1* mutants in species with simple leaves. While *AS1* negatively regulates the class I KNOX genes and promotes adaxial identity in all of the taxa shown, the relative importance of each of these roles varies by species. In *Antirrhinum,* the dominant phenotype appears to be loss of adaxial identity while in *Nicotiana* ectopic KNOX gene phenotypes and abaxialization phenotypes are seen (Waites and Hudson 1995; McHale and Koning 2004). In *A. thaliana* and maize, the phenotypes appear to be largely due to ectopic KNOX gene expression, but some abaxial/adaxial polarity defects can be seen when expression of AS1's binding partner AS2 is affected (Schneeberger et al. 1998; Byrne et al. 2000; Lin et al. 2003).

Simple leaves have a single flat lamina while compound leaves are composed of multiple regularly spaced blades, known as leaflets, arranged along or around a central main stem or rachis, which can take a number of different patterns (Fig. 1.5) (Reviewed in: Efroni et al. 2010)). In pinnate leaves, the leaflets are positioned along the sides of the rachis while in palmate leaves the leaflets are clustered at the tip of the rachis (Kim et al. 2003a). Palmate leaves are further categorized into peltately palmate leaves, in which the leaflets are attached around the entire circumference of the rachis terminus and non-peltately palmate leaves, in which no leaflet is present on the adaxial side of the rachis (Fig. 1.5) (Kim et al. 2003a). The basal portion of the rachis may also be termed the petiole while the individual stems bearing each leaflet are termed petiolules, although leaflets may also be sessile and lack any basal stem.

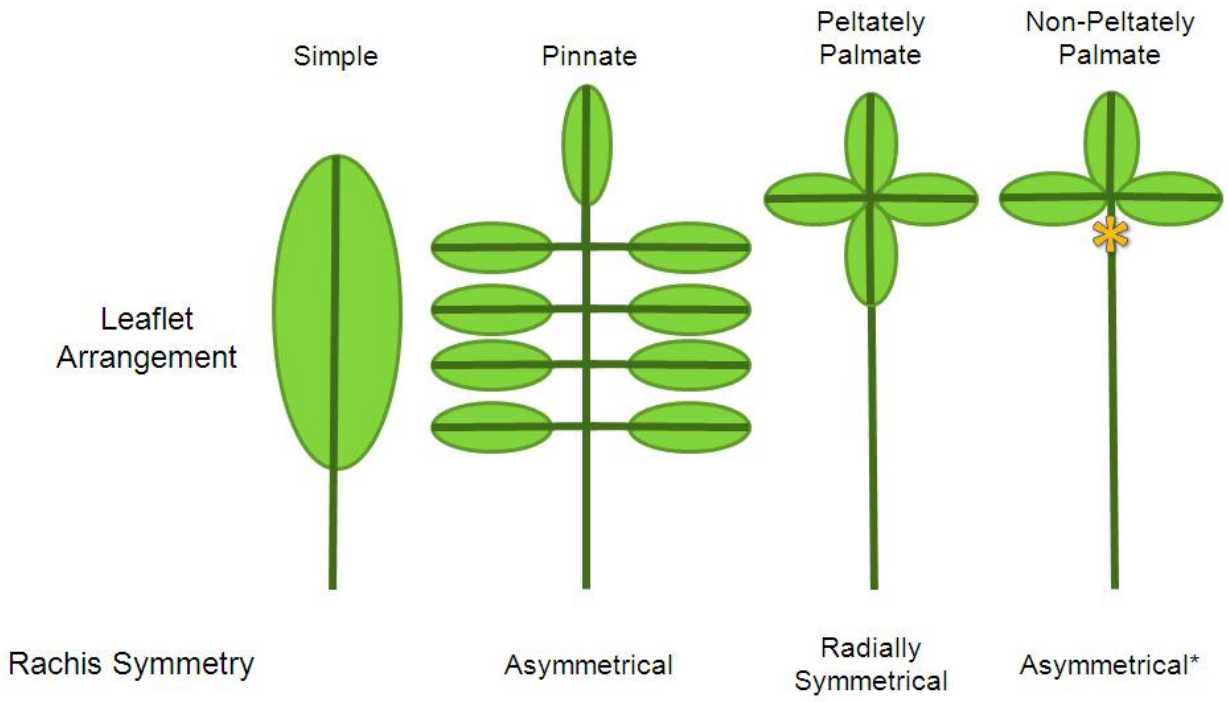


Figure 1.5: Leaflet arrangement and rachis symmetry in the major types of compound leaves. Pinnate leaves have leaflets arranged all around the rachis while in palmate leaves the leaflets are clustered at the tip of the rachis. In peltately palmate leaves the leaflets are arranged all the way around the rachis whereas in non-peltately palmate leaves the adaxial most leaflet is missing (orange asterisk). According to Kim et al. (2003a), pinnate and non-peltately palmate leaves have asymmetrical rachises with both adaxial and abaxial domains while peltately palmate leaf rachises are radially symmetrical.

In several compound-leafed models, *AS1* homologs appear to be involved in both initiating leaflets within the leaf primordium and in determining leaflet arrangement along or around the rachis. This role is likely related to the broadly conserved regulatory interactions between *AS1* and the class I KNOX genes. The class I KNOX genes may act to maintain indeterminacy in compound leaves and promote leaflet initiation

(Bharathan et al. 2002; Efroni et al. 2010). KNOX genes are expressed in the shoot apical meristem and down-regulated in incipient leaf primordia (P0), but subsequently turned back on in early leaf primordia around the time of leaflet initiation (Bharathan et al. 2002). Over expressing several class I KNOX results in increased leaflet number and branching within the leaf while reducing KNOX gene expression decreases leaf complexity (Hareven et al. 1996; Hay and Tsiantis 2006; Jasinski et al. 2007). In several compound leafed species, *AS1* homologs are expressed in both the leaf primordia and the SAM and their expression domain overlaps with that of the class I KNOX genes (Koltai and Bird 2000; Kim et al. 2003a). Analysis of several tomato mutants where expression of the KNOX genes *LeT6* and *TKN1* or the *AS1* homolog, *LePHAN* are disrupted suggests that as in simple leaves *LePHAN* negatively regulates*TKN1*. However, in contrast to simple leaves, these genes are expressed in the same domains and *LeT6* negatively regulates *LePHAN* (Kim et al. 2003b). Furthermore it appears that the expression of both *LeT6* and *LePHAN* is required for leaflet initiation and lamina expansion within the leaflets as down-regulation of one or the other of these genes results in leaves with reduced complexity that are often cup-shaped or needle-like (Kim et al. 2003a; Kim et al. 2003b).

AS1 may also help control the arrangement of the leaflets in compound leaves. In tomato, a proportion of *LePHAN* RNAi lines the leaves switch from being pinnate to peltately palmate. In these leaves the petiole is radialized and *LePHAN* expression is seen only in the distal tip of the primordia (Kim et al. 2003a). Kim et al (2003a) looked at the expression of *AS1* homologs in a number of species with compound leaves and

found that in species with pinnate and non-peltately palmate leaves the rachis has abaxial-adaxial asymmetry and *AS1* is expressed along the entire adaxial surface of the leaf primordia. However, in peltately palmate leaves the rachis is radially symmetric and *AS1* expression is restricted to the distal tip of the primordia where the leaflets initiate (Kim et al. 2003a). This suggests that the juxtaposition of abaxial-adaxial identity, in part controlled by *AS1*, is required not only for lamina expansion in the leaves and leaflets, but also for leaflet initiation (Kim et al. 2003a).

The role of *AS1* in compound leaf development and its interactions with class I KNOX genes may vary in some species. In *Cardamine hirsuta*, *ChAS1* is not expressed in the central zone of the SAM, but is instead seen in initiating leaf primordia and in the boundary region between the abaxial and adaxial domains in older leaves which is similar to the expression pattern seen in *A. thaliana* (Hay and Tsiantis 2006). Also like *A. thaliana*, *ChAS1* regulates *ChBP* but not *ChSTM* . While *ChSTM* is expressed in developing leaves and thought to promote leaflet initiation, *ChBP* is restricted to the SAM. In *chas1* mutants leaflet number is increased and the proximal-distal axes of the leaf appears compressed (Hay and Tsiantis 2006). Ectopic expression of *ChBP* is seen on the adaxial surface of these leaves suggesting that *ChAS1* may control leaf development by restricting *ChBP* expression (Hay and Tsiantis 2006). In pea, *Crispa (CRI),* the *AS1* homolog, is also only expressed in leaf primordia (Tattersall et al. 2005). However, the expression of the class I KNOX gene expression is restricted to the SAM and they are not thought to control leaflet initiation (Hofer et al. 1997; Tattersall et al. 2005). In *cri* mutants, ectopic KNOX gene expression is observed resulting in leaves

that are more complex than wild type leaves, abnormalities in vascular patterning, and ectopic stipules (Tattersall et al. 2005). Additionally abaxial-adaxial polarity appears to be disrupted in *cri* leaflets resulting in leaflets that are partially abaxialized and, occasionally, needle-like leaflets (Tattersall et al. 2005). However, *CRI* does not appear to control leaflet arrangement in pea as *cri* leaves remain pinnate (Tattersall et al. 2005).

To summarize, as in simple leaves, *AS1* regulates both adaxial identity and class I KNOX gene expression in species with compound leaves, but the relative importance of each of these functions varies between species resulting in very different phenotypes (Fig. 1.6). However, *AS1*-KNOX gene interactions are more complicated since they are often expressed in overlapping domains and both genes may be required for leaflet initiation in some species. *AS1* also controls abaxial-adaxial polarity in the rachis and leaflet arrangement in some species. However, tomato, pea, and *C. hirsuta* are all species with pinnately compound leaves. The function of *AS1* in palmately compound leaf development remains to be explored.

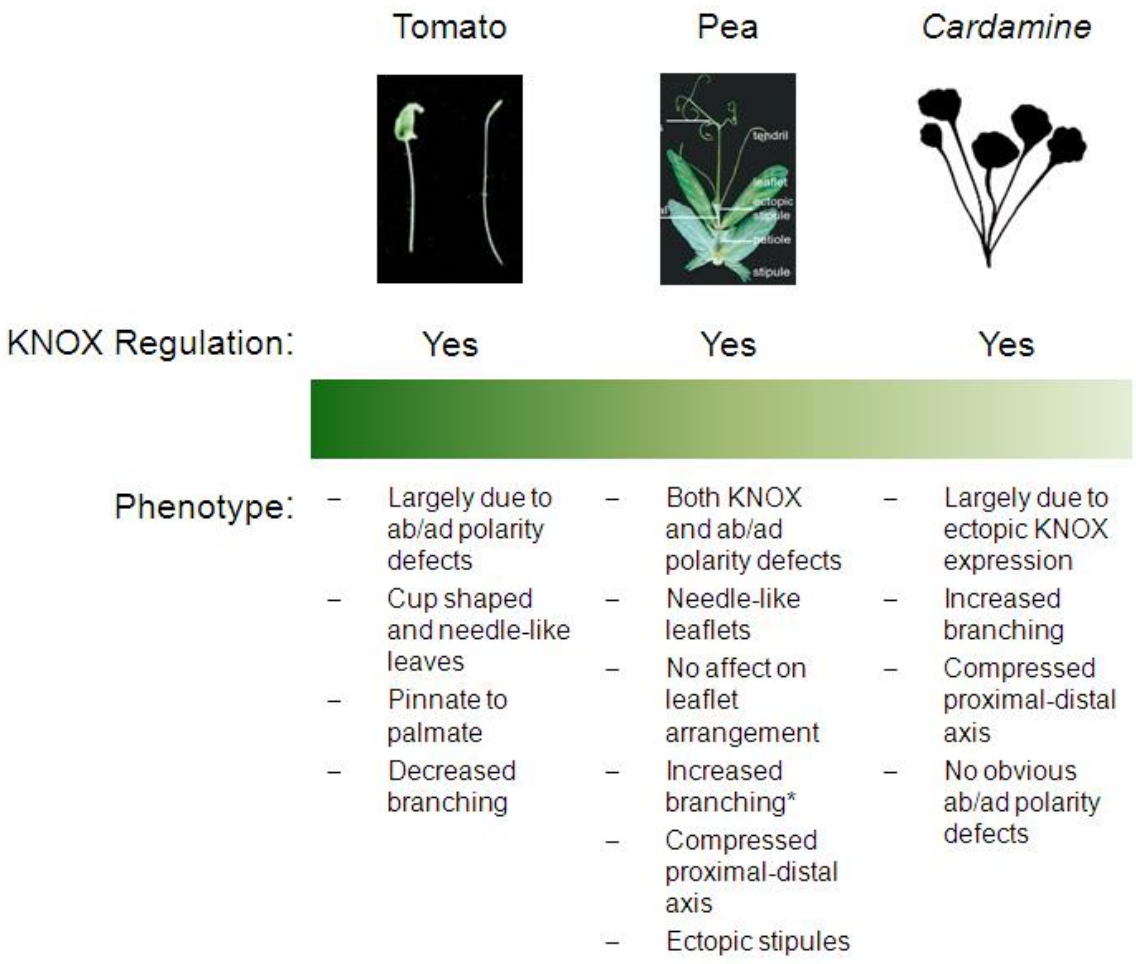


Figure 1.6 The gradient of phenotypes seen in *as1* mutants in species with complex leaves. While *AS1* negatively regulates the class I KNOX genes and promotes adaxial identity in all of the taxa shown, the relative importance of each of these roles varies by species. In tomato, the phenotypes observed appear to be largely due to loss of adaxial identity (Kim et al. 2003b). Without the juxtaposition of abaxial and adaxial identity, the parts of all of the leaves are radialized resulting in decreased branching and needle-like leaves. In pea, the phenotypes appear to be due to both ectopic KNOX expression and defects in abaxial-adaxial polarity. While individual leaflets are radialized in pea *as1* mutants, the leaf remains compound with no effect on leaflet arrangement (Tattersall et al. 2005). A slight increase in branching is observed which may be due to ectopic KNOX expression, however, KNOX genes to not control leaf

Figure 1.6 Continued: complexity in pea (Hofer et al. 1997). However, other classic ectopic KNOX gene phenotypes such as ectopic stipules and a compressed proximal-distal axis are observed. Finally in *C. hirsuta* the *as1* mutant phenotype appears to be largely due to ectopic KNOX gene expression. The leaves show many KNOX over-expression phenotypes including an increase in complexity and no loss of adaxial identity is observed (Hay and Tsiantis 2006).

1.3: *Aquilegia* as a Model System

In this study, we have sought to further our understanding of the evolution, expression, and function of PRC2 and *AS1* by studying their role in the emerging model system, *Aquilegia*. The genus *Aquilegia,* which has been the subject of ecological, evolutionary and genetic studies for over 50 years, is of interest for a number of reasons (Reviewed in: Hodges and Kramer 2007). First, *Aquilegia* is a tractable model system due to its small genome (n=7, approximately 300 Mbp) and a number of genetic and genomic tools, including an extensive EST database and the recently sequenced *Aquilegia coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) (Reviewed in: Kramer 2009). The reverse genetic tool, virus-induced gene silencing (VIGS), has been optimized in several *Aquilegia* species, making it possible to conduct functional studies (Gould and Kramer 2007; Kramer et al. 2007; Sharma et al. 2011; Pabón-Mora et al. 2013; Sharma and Kramer 2013). Second, as a member of the order Ranunculales, an early diverging lineage of the eudicotyledonous flowering plants that arose before the radiation of the core eudicots, it represents a rough phylogenetic midpoint between *A. thaliana* and model systems in the grasses (Fig. 1.5) (Reviewed in: Kramer and Hodges 2010). *Aquilegia* has undergone a recent adaptive radiation,

resulting in low sequence variation and a high degree of fertility between species which allows the use of multiple different species as models as well as the use of interspecific crosses. Finally, *Aquilegia* has a number of interesting morphological and physiological features, including vernalization-based control of flowering that is thought to represent what is likely to be an independent derivation of vernalization response relative to *A. thaliana* and the grasses (Ballerini and Kramer 2011) and independently evolved compound leaves.

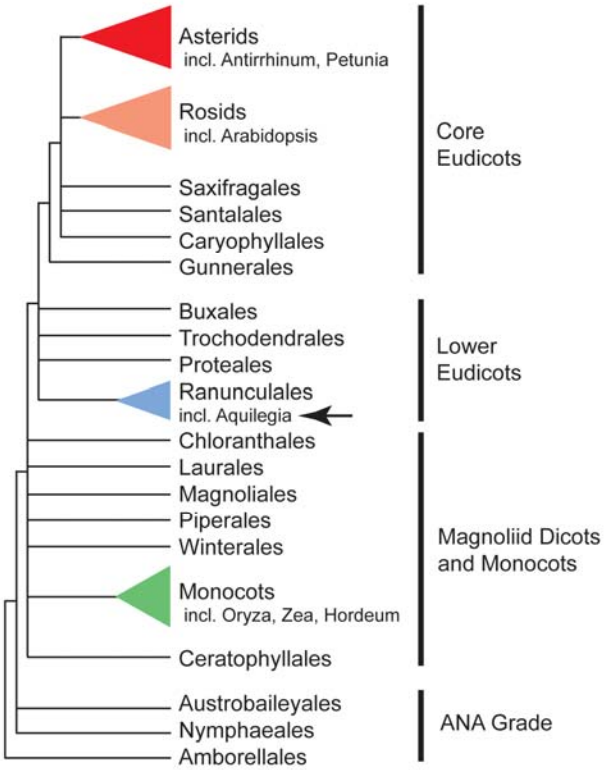


Figure 1.7: Simplified angiosperm phylogeny. As an early diverging lineage of the eudicotyledonous flowering plants that arose before the radiation of the core eudicots (a group that includes *A. thaliana)*, occupies an important phylogenetic position serving as a rough phylogenetic midpoint between *A. thaliana* and model systems in the grasses (Reviewed in: Kramer and Hodges 2010).

In Chapter 2, we have performed broad identification of chromatin remodeling homologs in the recently sequenced *A. coerulea* genome with more detailed study of PRC2 and VEL PHD homologs. The strongly vernalization-responsive species *A. vulgaris* was further utilized to determine broad expression patterns over a range of tissue types and developmental stages. Lastly, we used interspecific crosses and naturally occurring polymorphism to investigate patterns of imprinting in the paralogous *AqCLF* and *AqSWN* loci. This work lays the foundation for future studies of epigenetic modification in the lower eudicot model *Aquilegia* and provides sequence data for broadly evolutionary studies of numerous gene families.

In Chapter 3, we have examined the functions of PRC2 members in lateral organ development in *A. coerulea*. Using VIGS to knock down the expression of *AqFIE* and *AqEMF2* in unvernalized and vernalized *Aquilegia coerulea* 'Origami' plants, we find that PRC2 plays a role in leaf and floral organ development, particularly via down-regulation of the floral MADS box genes. This has allowed us to identify PRC2 targets that appear to be conserved between *A. thaliana* and *Aquilegia* as well as some novel PRC2-regulated pathways.

Finally, in Chapter 4, we characterize the expression patterns of *AS1* and *AS2* homologs in *A. coerulea* and to examine the knock-down phenotype of *AqAS1*. These analyses, together with studies of candidate interacting loci, provide much greater insight into both the conservation of *AS1* lineage function and the novel aspects of leaf development that may be at work in *Aquilegia*.

Combined, these studies offer some insight into the role of two important gene regulatory mechanisms in *Aquilegia* lateral organ development. Most interestingly, these datasets suggest that contrary to the widely held model, class I KNOX genes are neither necessary nor sufficient for leaf complexity in *Aquilegia*.

Chapter 2:

Characterization of *Aquilegia* Polycomb Repressive Complex 2 homologs reveals absence of imprinting.

This chapter is reformatted from the published version:

Gleason, E. J. and E. M. Kramer (2012). "Characterization of Aquilegia Polycomb Repressive Complex 2 homologs reveals absence of imprinting." Gene 507(1): 54-60.

2.1: Introduction

The last common ancestor of plants and animals lived approximately 1.6 billion years ago, before the evolution of multicellular organisms (Reviewed in: Meyerowitz 2002). Thus, multicellularity most likely arose independently in these two groups and, accordingly, many aspects of their development are very different. However, in both lineages the maintenance of proper gene expression in differentiated cells is essential for the development of multicellularity. Gene expression is maintained via a process of cellular memory known as epigenetic regulation (Reviewed in: Holliday 1994; Russo et al. 1996; Feil 2008). Many proteins involved in epigenetic maintenance of gene expression are highly conserved between plants and animals and appear to function in a remarkably similar manner (Reviewed in: Pien and Grossniklaus 2007; Whitcomb et al. 2007; Köhler and Hennig 2010).

One key example is the Polycomb Group (PcG), a set of proteins with important and deeply conserved functions in epigenetic silencing. These proteins were first discovered in *Drosophila melanogaster* as repressors of the HOX genes (Lewis 1978). In animals, the PcG proteins form several multimeric complexes each with distinct functions in epigenetic silencing. Two such complexes, known as Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2), function in tandem to repress

gene expression (Schuettengruber et al. 2007). The PRC2 contains four core proteins; the histone methyltransferase *Enhancer of Zeste* (*E(z))* and three other proteins thought to enhance PRC2 binding to nucleosome (Nekrasov et al. 2005); *Suppressor of Zeste 12 (Su(z)12), Extra Sex Combs (ESC)*, and *Multi-Copy Suppressor of IRA 1 (MSI1*) (Reviewed in: Pien and Grossniklaus 2007). The main function of the PRC2 complex appears to be trimethylation of lysine 27 of histone H3 (H3K27), a histone modification known to suppress gene expression (Schubert et al. 2006). The PRC1 contains several core components and binds to the H3K27 trimethylation deposited by PRC2 and stably represses gene expression (Reviewed in: Schuettengruber et al. 2007).

Only the Polycomb Repressive Complex 2 (PRC2) is thought to be homologous between plants and animals (Reviewed in: Schuettengruber et al. 2007; Whitcomb et al. 2007). Recently a complex has been identified in A. thaliana that may have PRC1-like function (Xu and Shen 2008; Bratzel et al. 2010), but this complex appears to include both RING finger proteins, similar to the animal PRC1 complex, as well as both LHP1, a plant homolog of the animal protein HP1 that is not found in the animal PRC1 complex, and EMF1, a plant specific protein (Calonje et al. 2008; Xu and Shen 2008; Exner et al. 2009; Bratzel et al. 2010; Beh et al. 2012). Thus, it appears that while the PRC2 complex members are genetically homologous between multicellular organisms, the plant protein complex that plays a functionally analogous role to PRC1 is largely composed of subunits that are not homologous to members of the animal PRC1 complex. In some plant species, including rice and *Arabidopsis thaliana,* duplications in

the core PRC2 proteins allow these species to form PRC2's with distinct developmental functions (Whitcomb et al. 2007; Luo et al. 2009).

Recent studies have shown that the PRC2 is involved in developmental transitions in a number of plant species. In the plant model system *A. thaliana*, PRC2s function in many processes including endosperm development, early repression of flowering to allow proper vegetative development, the eventual transition to flowering, and flower organogenesis (Goodrich et al. 1997; Gendall et al. 2001; Yoshida et al. 2001; Kohler et al. 2003b). In rice, the mutant phenotype of *OsEMF2b*, suggests that the PRC2 complex may play a role in floral induction under long days, flower development, and suppressing cell divisions in the unfertilized ovule (Luo et al. 2009). The PRC2 may also regulate the induction of flowering in response to vernalization in barley. ChIP analysis of the barley floral promotion locus *VERNALIZATION 1* (*VRN1*) before and after vernalization showed that regulatory regions contained differential levels of H3K27 trimethylation, the histone modification deposited by the PRC2 complex (Oliver et al. 2009). This suggests that the PRC2 complex may function in floral induction in barley as well (Oliver et al. 2009). In the moss species, *Physcomitrella patens*, deletion of the PRC2 genes *PpCLF* and *PpFIE* induces sporophyte-like development and gene expression in the gametophyte, indicating that PRC2-dependent remodeling may be required for the switch from gametophyte to sporophyte development (Mosquna et al. 2009; Okano et al. 2009).

Consistent with these common roles in regulating life stages and tissue identity, another component of PRC2 function in flowering plants is a role in differential imprinting of loci in the maternal and paternal genomes of developing embryos and endosperm, the latter being a nutritive tissue containing two maternal and one paternal genomic complements (Reviewed in: Baroux et al. 2002). Furthermore, members of the PRC2 complex itself have been found to be imprinted in *A. thaliana* and several grasses (Kinoshita et al. 1999; Springer et al. 2002; Guitton et al. 2004; Luo et al. 2009). In the cases of *MEDEA (MEA)*, *FERTILIZATION INDEPENDENT SEED 2* (*FIS2)*, and *FERTILIZATION INDEPENDENT ENDOSPERM (FIE)*, the loci are imprinted in the endosperm such that the maternal copies are expressed while the paternal copies are silenced (Guitton et al. 2004). Recent work has demonstrated that a maize E(z)-like gene (*Mez1*), maize *ZmFIE1*, and rice *OsFIE1* are similarly imprinted in the endosperm, suggesting that PcG imprinting may be a common theme in endosperm development (Springer et al. 2002; Haun et al. 2007; Luo et al. 2009). However, it is unclear if this role is conserved between the grasses and *A.thaliana* or if the PRC2 complex has been recruited several times independently for this function.

In both plants and animals, PRC2s are thought to associate with other proteins that help recruit them to specific loci (Reviewed in: Köhler and Hennig 2010; Margueron and Reinberg 2011). In *A. thaliana*, members of a plant specific group known as the VIL (VIN3-like) or VEL PHD family have been shown to associate with the PRC2 complex and seem to be required for PRC2 repression of the floral repressor, *FLC,* during and after vernalization (Sung et al. 2006; Greb et al. 2007; De Lucia et al. 2008). Intriguingly,

VEL PHD homologs are also induced by vernalization in wheat, despite the fact that the grasses evolved their cold response independently (Fu et al. 2007a). It remains to be determined, however, whether these wheat genes are actually functioning in the floral promotion pathway.

Here we examine the evolution and expression of the PRC2 and VEL PHD families in the emerging model system, *Aquilegia*. The genus *Aquilegia* has been the subject of ecological, evolutionary and genetic studies for over 50 years (Reviewed in: Hodges and Kramer 2007). *Aquilegia* is of interest for a number of reasons. First, *Aquilegia* has a small genome (n=7, approximately 300 Mbp) with a number of genetic and genomic tools, including an extensive EST database and the recently sequenced *Aquilegia coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) (Reviewed in: Kramer 2009). Second, as a member of the order Ranunculales, an early diverging lineage of the eudicotyledonous flowering plants that arose before the radiation of the core eudicots, it represents a rough phylogenetic midpoint between *A. thaliana* and model systems in the grasses (Reviewed in: Kramer and Hodges 2010). Additionally *Aquilegia* has a number of interesting morphological and physiological features including vernalization-based control of flowering which is thought to represent what is likely to be an independent derivation of vernalization response relative to *A. thaliana* and the grasses (Ballerini and Kramer 2011). Finally, *Aquilegia* has undergone a recent adaptive radiation, resulting in low sequence variation and a high degree of fertility between species. This allows the use of multiple different species as models as well as the use of interspecific crosses to test phenomena such as imprinting.

In the current study, we have performed broad identification of chromatin remodeling homologs in the recently sequenced *A. coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) with more detailed study of PRC2 and VEL PHD homologs. The strongly vernalization-responsive species *A. vulgaris* was further utilized to determine broad expression patterns over a range of tissue types and developmental stages. Lastly, we used interspecific crosses and naturally occurring polymorphism to investigate patterns of imprinting in the paralogous (*E(z)*) homologs, *AqCLF* and *AqSWN* loci. This work lays the foundation for future studies of epigenetic modification in the lower eudicots model *Aquilegia* and provides sequence data for broad evolutionary studies of numerous gene families.

2.2: Methods

Gene cloning

In order to identify genes of interest, BLAST searches (Altschul et al. 1990) of the *Aquilegia* DFCI Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=*Aquilegia*) and the *Aquilegia coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) were performed using the sequences of our genes of interest from *A. thaliana* or, in a few cases, from *Vitis vinifera*.

In the cases of *AqFIE, AqEMF2*, and *AqCLF,* BLAST searches did not identify the full length sequence, so 3'and 5' Rapid Amplification of cDNA Ends (RACE) was used to

determine the complete sequence. The targeted loci were amplified from a mix of cDNA prepared from RNA isolated from young leaves and primers designed based on the fragments obtained above (see Appendix 1 for primer sequences). 5' RACE followed the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 protocol (Invitrogen, Carlsbad, CA). 3' RACE was performed as described in Kramer et al. (2003). Fragments were cloned using the TOPO-TA Cloning Kit and TOP10 competent cells (Invitrogen, Carlsbad, CA) and several clones per cloning reaction were sequenced using Big Dye v3.1 (Life Technologies Corporation, Carlsbad, CA).

In the case of *AqSWN*, *AqVIN3A*, *AqVIN3B*, and *AqVRN5,* BLAST searches did not identify an EST or predicted an open reading frame, so a BLAST search of the *Aquilegia coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) was used to identify regions that showed similarity to the query sequence. The Soft Berry FGENESH program (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) was then used to predict open reading frames for the loci. cDNA sequences were confirmed using specific primers designed for internal Reverse Transcriptase PCR (RT-PCR) as described in Kramer et al. (2003) as well as 5' RACE for *AqVIN3B* and 3' RACE for *AqSWN* as described above. All new sequences are deposited in Genbank under accession numbers JN944598- JN944605 (See Appendix 2).

Phylogenetic analysis

For all gene trees, homologs to the PRC2 genes and VEL PHD family were identified for a variety of land plant taxa by using the BLAST algorithm to search GenBank, the DFCI Plant Gene Index (http://compbio.dfci.harvard.edu/tgi/plant.html) , the Selaginella genomics database (http://xselaginella.genomics.purdue.edu/), and ChromDB (http://www.chromdb.org/) (Gendler et al. 2008) or through literature searches.

For all datasets, amino acid sequences were initially aligned using Clustal W and then adjusted by hand using MacVector (Cary, North Carolina). Maximum likelihood analysis was completed using RAxML (Stamatakis et al. 2008) as implemented by the CIPRES Science Gateway (http://www.phylo.org/portal2/login!input.action) (Miller et al. 2010). The model of amino acid evolution used was the default JTT. Bootstrap values are presented at all nodes with greater than 50% support while nodes with less than 50% support are collapsed.

Quantitative real-time PCR

To asses expression of the PRC2 genes and VEL PHD family throughout the life cycle of *A. vulgaris*, the following tissue was collected from *A. vulgaris* plants: whole seedlings at the cotyledon, 1-3 leaf, and 6-8 leaf stages; leaves from 8-12 leaf stage plants; 8-12 leaf stage meristems (before vernalization); meristems subjected to 4 weeks of cold treatment at 4°C (during vernalization); meristems subjected to 8 weeks of cold treatment then removed to 18°C (after vernalization); inflorescence meristems; anthesis stage sepals, stamens and carpels; and developing fruits. At each stage, samples from

three to five different plants were collected and pooled. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA was treated with Turbo DNase (Ambion, Austin, TX) and cDNA was synthesized from 10 µg of total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT) primers.

Quantitative Real Time PCR (qRT-PCR) reactions were carried out using PerfeCTa SYBR Green FastMix Low Rox (Quanta Biosciences, Gaithersburg, MD) and analyzed in the Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA). Each 20 µl reaction included 4 µl of cDNA that had been diluted 1:5 and had a final primer concentration of 0.25 nmol/µL. A list of primers is included in Appendix 1. Standard curves were run for all primer pairs to ensure high efficiency. The annealing temperature of all genes was 60° C with a 30 second extension. For each data point, three technical replicates were analyzed. *AqIPP2* (isopentyl pyrophosphate:dimethylallyl pyrophosphate isomerase) expression was used for normalization.

Assessment of PRC2 Homolog Imprinting in *Aquilegia* Endosperm

In order to determine if any members of the *Aquilegia* PRC2 complex are imprinted in the endosperm, genetic polymorphisms between interfertile species of *Aquilegia* were used. Several individual *A. canadensis* and *A. vulgaris* plants were obtained and total RNA was extracted from the young leaves of these plants using the RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from 5ug RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The 3' UTR of *AqSWN* and *AqCLF* were amplified by RT-PCR using Platinum Taq (Invitrogen, Carlsbad, CA) with specific

primers (Appendix 1) and purified using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) followed by column purification using the PCR Purification Kit (Qiagen, Valencia, CA). These gene fragments were then directly sequenced using Big Dye v3.1 (Life Technologies Corporation, Carlsbad, CA) and sequences were aligned with Clustal W using MacVector (Cary, North Carolina). This allowed the identification of single nucleotide polymorphisms (SNPs) representing restriction polymorphisms that could distinguish one of the *A. vulgaris* plants from one of the *A. canadensis* plants.

Several flowers on each of these plants were emasculated and reciprocal crosses were performed. Seeds were collected when the seed coat was dark green and the endosperm had just cellularized (approximately a week after fertilization). At this stage, the *Aquilegia* embryo is approximately 1 mm in length out of a total seed length of 4 mm and is tightly positioned at the micropylar end of the seed. Seeds were bisected horizontally to separate the embryo containing half from the endosperm-only half and these separate samples were pooled to obtain 100mg of material for each.

RNA was extracted from the seeds using the method described by Vicient and Delseny (1999) with some modifications. The RNA was only extracted once in Phenol and the phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol steps were eliminated. The aqueous phase was then collected and separated into two 1.6 ml microcentrifuge tubes. A 0.1x volume of 3M sodium acetate and a 1.5x volume of ethanol was added to each tube and the mixture was stored overnight at -20^{o} C. The tubes were then spun at 13,000 RPM for 30min at 4^{o} C and the pellet was resuspended

in 200µl of Lysis/Binding Solution from the RNAqueous kit (Ambion, Austin, TX) which was then used to further purify the RNA. RNA was treated with Turbo DNase (Ambion, Austin, TX).

cDNA was prepared from this RNA and the 3' end of *AqSWN* and *AqCLF* were amplified using the same methods as described above for parental leaves. For each digest, several RT-PCR amplifications were pooled before purification in order to obtain an adequately concentrated sample. *AqSWN* gene fragments from both seed halves and parental leaves were digested with Bpu10I in Buffer 3 (New England BioLabs, Ipswich, MA) for 2 hours at 37^{o} C and run on a 2% agarose gel and visualized with Ethidium Bromide. *AqCLF* fragments from seeds and maternal leaves (control) were digested with AcuI in Buffer 4 and 40 µM S-adenosylmethionine (New England BioLabs, Ipswich, MA) for 16 hours at 37^{o} C and run on a 2% agarose gel and visualized with Ethidium Bromide.

2.3: Results and Discussion

Homologs of the PRC2 and the VEL PHD family in the *Aquilegia* Genome

We used a variety of bioinformatic approaches to identify PRC2 and VEL PHD homologs from the *Aquilegia coerulea* genome. Similar to *A. thaliana, A. coerulea* only has one ESC homolog, *AqFIE* (Fig. 2.1).

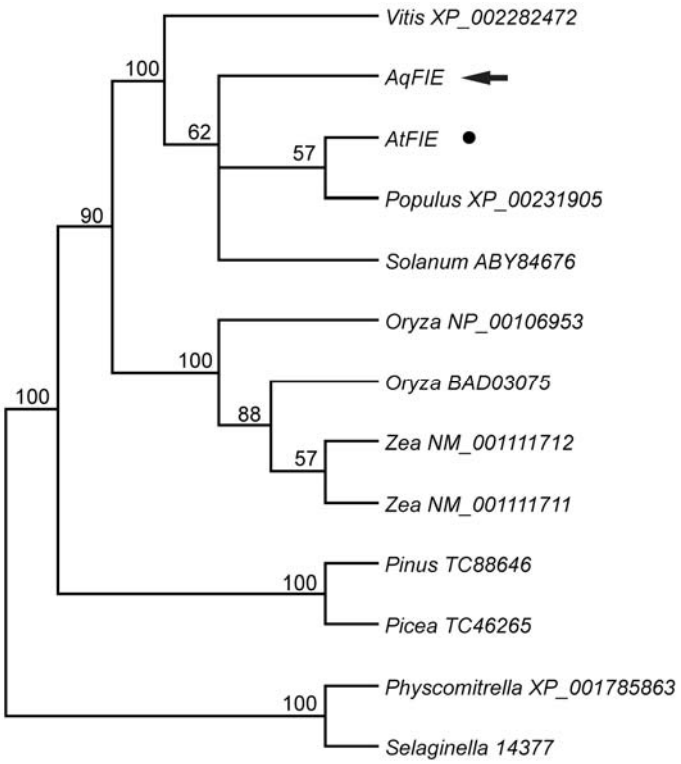


Figure 2.1: Phylogenetic analysis of homologs of FIE in the land plants. Maximum likelihood (ML) analysis of Extra Sex Combs (ESC)/*FERTILIZATION INDEPENDENT ENDOSPERM (FIE)* homologs with ML bootstrap values shown at the nodes. Nodes with <50 support are collapsed. At = *Arabidopsis thaliana*; Aq=*Aquilegia*. *A. thaliana* sequences are indicated with a dot; *Aquilegia*, with an arrow.

Of the three identified homologs of *Multi Copy Suppressor of IRA* (*MSI*) (Fig. 2.2), one, *AqMSI1*, appears to be most similar to *MSI1* in *A. thaliana,* which has been shown to associate with the PRC2 (De Lucia et al. 2008). The other two loci group with *A. thaliana MSI2* and *MSI3* (*AqMSI2*) or *A. thaliana FVE* and *MSI5* (*AqFVE*) (Hennig et al. 2003).

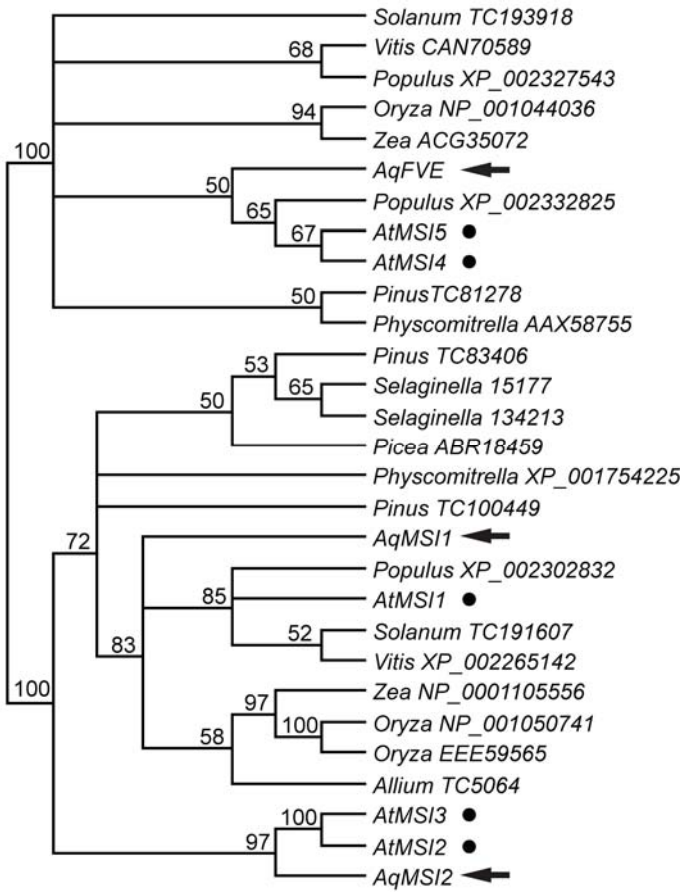


Figure 2.2: Phylogenetic analysis of homologs of MSI1 in the land plants. Maximum likelihood (ML) analysis of homologs of the yeast protein Multi Copy Suppressor of IRA (MSI1) with ML bootstrap values shown at the nodes. Nodes with <50 support are collapsed. At=*Arabidopsis thaliana*; Aq=*Aquilegia*. *A. thaliana* sequences are indicated with a dot; *Aquilegia*, with an arrow.

While *A. thaliana* has four homologs of Su(z)12 - *EMF2*, *VRN2*, *FIS2*, and *AT4G16810* (Chen et al. 2009) we only identified one homolog in *A. coerulea*, *AqEMF2* (Fig. 2.3). This finding is consistent with other phylogenetic analyses of the VEFS domain containing proteins in plants in which Chen et al. (2009) claim that *VRN2*, *FIS2,* and *AT4G16810* are derived from rosid-specific duplication events, albeit with no statistical

support . Note that although *AqEMF2* is not orthologous to *A. thaliana EMF2*, we used the nomenclature of Cheng et al. (2009) in designating it *AqEMF2*.

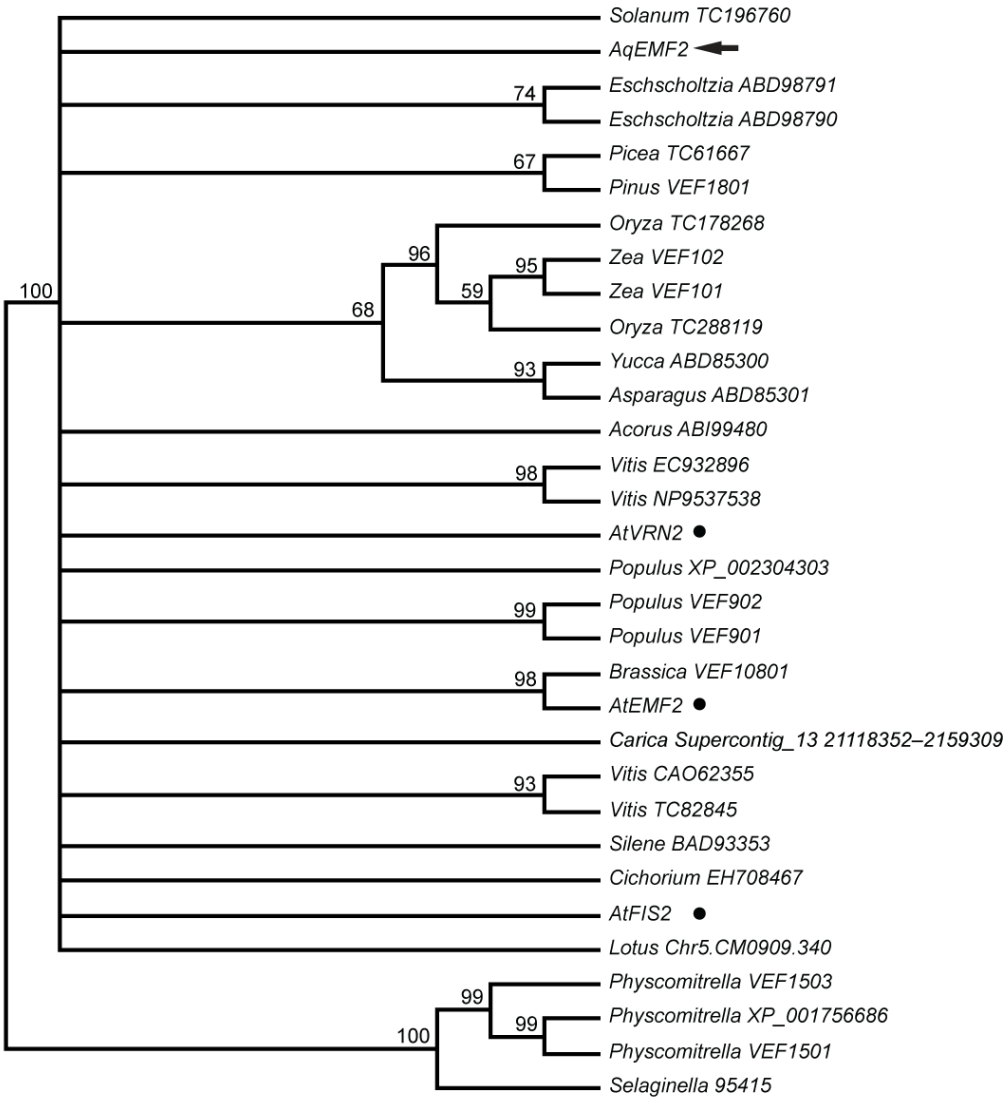


Figure 2.3: Phylogenetic analysis of VEFS box proteins in the land plants. Maximum likelihood (ML) analysis of VEFS box containing proteins including *VERNALIZATION 2 (VRN2), EMBRYONIC FLOWER 2 (EMF2),* and *FERTILIZATOIN INDEPENDENT SEED 2 (FIS2)* with ML bootstrap values shown at the nodes. Nodes with <50 support are collapsed.

Figure 2.3 Continued: At=*Arabidopsis thaliana*; Aq=*Aquilegia*. *A. thaliana* sequences are indicated with a dot; *Aquilegia*, with an arrow.

We recovered two E(z) homologs in *A. coerulea*, one that belongs to the *CLF* clade, *AqCLF,* and one from the *SWN* clade, *AqSWN* (Fig. 2.4)*. A. thaliana* has three E(Z)-like genes, *CLF, SWN,* and *MEA* (Baumbusch et al. 2001), however, phylogenetic analysis suggests that *MEA* is a product of a Brassicaceae-specific duplication of *SWN* (Spillane et al. 2007).

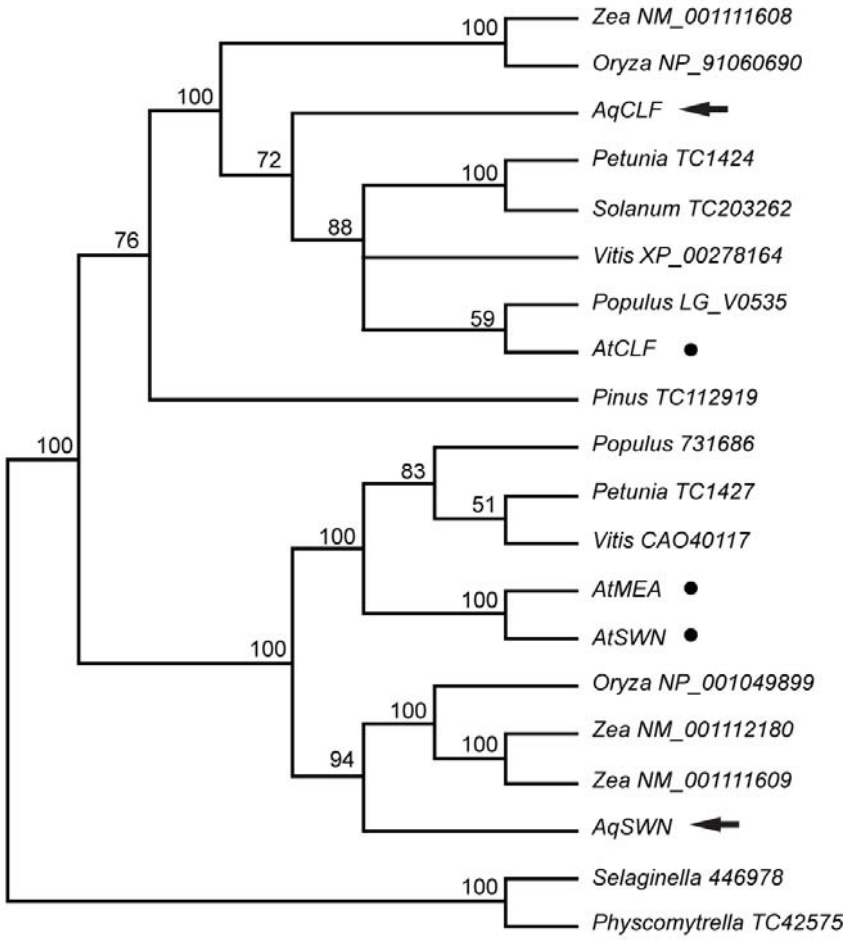


Figure 2.4: Phylogenetic analysis of homologs of Enhancer of Zeste in the land plants. Maximum likelihood (ML) analysis of the SET domain containing Enhancer of Zeste E(z)

Figure 2.4 Continued: homologs including *CURLY LEAF (CLF), SWINGER (SWI),* and *MEDEA (MEA)* with ML bootstrap values shown at the nodes. Nodes with <50 support are collapsed. At=*Arabidopsis thaliana*; Aq=*Aquilegia*. *A. thaliana* sequences are indicated with a dot; *Aquilegia*, with an arrow.

Together, our analysis of the *A. coerulea* PRC2 homologs suggests that relative to other model systems like rice and *A. thaliana*, *Aquilegia* has a simpler compliment of PRC2 homologs with no recent duplications.

We also searched for homologs of the VEL PHD gene family, which include co-factors of PRC2 (Greb et al. 2007; De Lucia et al. 2008). These could not be identified from available annotated genes so we used a combination of DNA sequence similarity and gene prediction software (http://linux1.softberry.com/berry.phtml?topic=fgenesh& group=programs&subgroup=gfind) to identify four *A. coerulea* VEL PHD genes (Fig. 2.5). Our phylogenetic analysis shows that there are several clades within the angiosperm VEL PHD family. The first contains *A. thaliana VRN5* and one *A. coerulea* gene, *AqVRN5.* A second clade contains several *A. thaliana* genes including *VEL1, 2,* and *3* and *VIN3* as well as two genes from *A. coerulea* termed *AqVIN3A* and *AqVIN3B*. A third clade contains one *A. coerulea* gene, *AqPHD1*, in addition to representatives from *Vitis* and rice but no apparent *A. thaliana* homolog. This study indicates that while ancient duplications established these three main lineages, the *A. thaliana* gene family was strongly influenced by recent duplications that generated the four *VIN3/VEL1-3* loci.

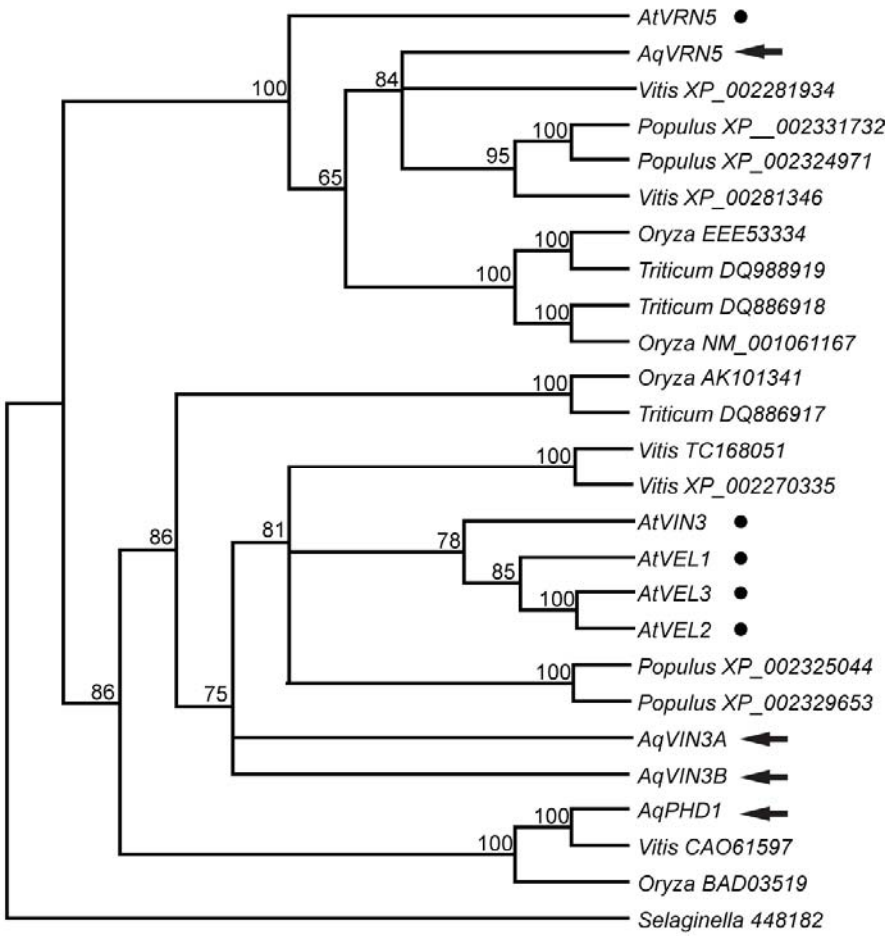


Figure 2.5: Phylogenetic analysis of the VEL PHD family. Maximum likelihood (ML) analysis of the VEL PHD family with ML bootstrap values shown at the nodes. Nodes with <50 support are collapsed. At=*Arabidopsis thaliana*; Aq=*Aquilegia*. *A. thaliana* sequences are indicated with a dot; *Aquilegia*, with an arrow.

In a further effort to annotate epigenetic loci from *A. coerulea*, other homologs of major gene lineages, including the PAF1 and SWR1 complexes as well as several genes thought to have PRC1-like function in plants, are shown in Table 1. These are purely bioinformatic identifications, however, unlike the PRC2 and VEL PHD homologs, which were confirmed using RT-PCR.

Table 1: *Aquilegia* orthologs of some *A. thaliana* loci involved in epigenetic regulation. Every line corresponds to what appears to be a single *Aquilegia* locus with a unique genome location. Some loci are represented by more than 1 EST.

Protein Family	Arabidopsis Gene	Aquilegia Gene	Aquilegia Locus Name	Aquilegia EST	Genome Location:		
					Scaffold	Approximate ATG Location	Strand
PRC2 Complex	CLF	AqCLF	AcoGoldSmith_v1.000712m.g	TC 32746, 4516_0_CCPG2564.g1_CCPF_CCPG, 4516_0_CCPG2564.b1_CCPF_CCPG	15	74820	Plus
	SWN	AqSWN	AcoGoldSmith_v1.013905m.g	None	3	7237059	Plus
	EMF2	AqEMF2	AcoGoldSmith_v1.009885m.g	TC27699	53	348873	Plus
	FIE	AqFIE	None	TC30447	15	1652603	Plus
	MSI1	AqMSI1	None	TC23711	26	1551886	Plus
PRC1-Like Function	LHP1/TFL2	AqLHP1	AcoGoldSmith_v1.005765m.g	TC21769	31	1046312	Plus
	VRN1		AcoGoldSmith_v1.020236m.g	TC21097	10	2058960	Plus
			AcoGoldSmith_v1.024292m.g	TC21097	11	93232	Minus
	EMF1	AqEMF1	AcoGoldSmith_v1.000332m.g	None	1	4132791	Minus
	RING1 A/B	AqRING1	AcoGoldSmith_v1.010607m.g	2059_1_CCPF_CCPG	36	1524946	Plus
Other P55 Related Proteins	MSI2/3	AqMSI2	AcoGoldSmith_v1.006377m.g	TC22385	7	138754	Plus
	MSI4/5/FVE	AqFVE	AcoGoldSmith_v1.004753m.g	TC21304	1	614606	Plus
VEL PHD PTHR21736	VIL1/VRN5	AqVRN5	AcoGoldSmith_v1.001521m.g	DT743202, 889_1_CCPF_CCPG	1	6674791	Plus
	VIN3	AqVIN3A	AcoGoldSmith_v1.022649m.g	None	81	254740	Minus
		AqVIN3B	AcoGoldSmith_v1.027813m.g, AcoGoldSmith_v1.013019m.g	TC30871, DR937521, 2948_1_CCPF_CCPG	96	264798	Plus
	None	AqPHD1	AcoGoldSmith_v1.016393m.g	None	10	1672981	Minus

Expression Analysis of the PRC2 and VEL PHD homologs in *A. vulgaris*

We characterized the expression of the five putative members of the PRC2 in *A. vulgaris* as well as the three VEL PHD genes most similar to the *A. thaliana* genes with known function. Tissue was collected at different stages throughout the life cycle of *Aquilegia vulgaris* and qRT-PCR was used to assess their expression. Three technical replicates were analyzed for each primer set on each sample and the data was normalized relative to the expression of the housekeeping gene *AqIPP2*.

We found that most PRC2 homologs are expressed at similar levels in all tissues and life stages sampled (Fig. 2.6A). One notable exception is *AqMSI1* whose expression level increases almost 10 fold in apical meristems during vernalization and almost 8 fold in early inflorescence meristems as compared to its expression at the cotyledon stage (Fig. 2.6A). *MSI1* homologs in other species are known to participate in other chromatin remodeling complexes so this increase in expression may be due to parallel functions and could reflect the large amount of chromatin remodeling necessary to complete these critical developmental transitions (Kohler et al. 2003b). We also observed a small increase in *AqFIE* expression in both the fruits and the carpels, however, it is unclear if this increase in expression is functionally relevant because the expression levels of the other PRC2 members remain low in these tissues. Consistent the role that the PRC2 complex is hypothesized to play throughout development we conclude that the entire complex is present at consistent levels at all developmental stages. In *A. thaliana,* the PRC2 gene, *VRN2* does not have a very dynamic expression pattern during vernalization, despite the important role it plays at this stage (Gendall et al. 2001).

The VEL PHD finger family members, *AqVIN3A, AqVIN3B,* and *AqVRN5* are also expressed throughout *A. vulgaris* development (Fig. 2.6B). *AqVIN3A* and *AqVRN5* peak in expression in the inflorescence and *AqVIN3B* expression is high at this stage as well. *AqVIN3B* expression peaks in the stamens while *AqVIN3A* is particularly low in this tissue. We cannot rule out that *A. vulgaris* VEL PHD proteins play a role in vernalization but, if they do, it does not appear to be mediated by specific expression patterns as with VIN3 in *A. thaliana* and wheat (Sung and Amasino 2004; Fu et al. 2007a). However, VEL PHD family members may also be involved in other aspects of plant development. For example, a rice VEL PHD gene, *LEAF INCLINATION 2,* has been show to repress cell divisions in the region between the leaf blade and leaf sheath known as the collar and thus contribute to leaf angle (Zhao et al. 2010). It may be interesting to further investigate the role of the VEL PHD genes in aspects of *Aquilegia* development beyond flowering time.

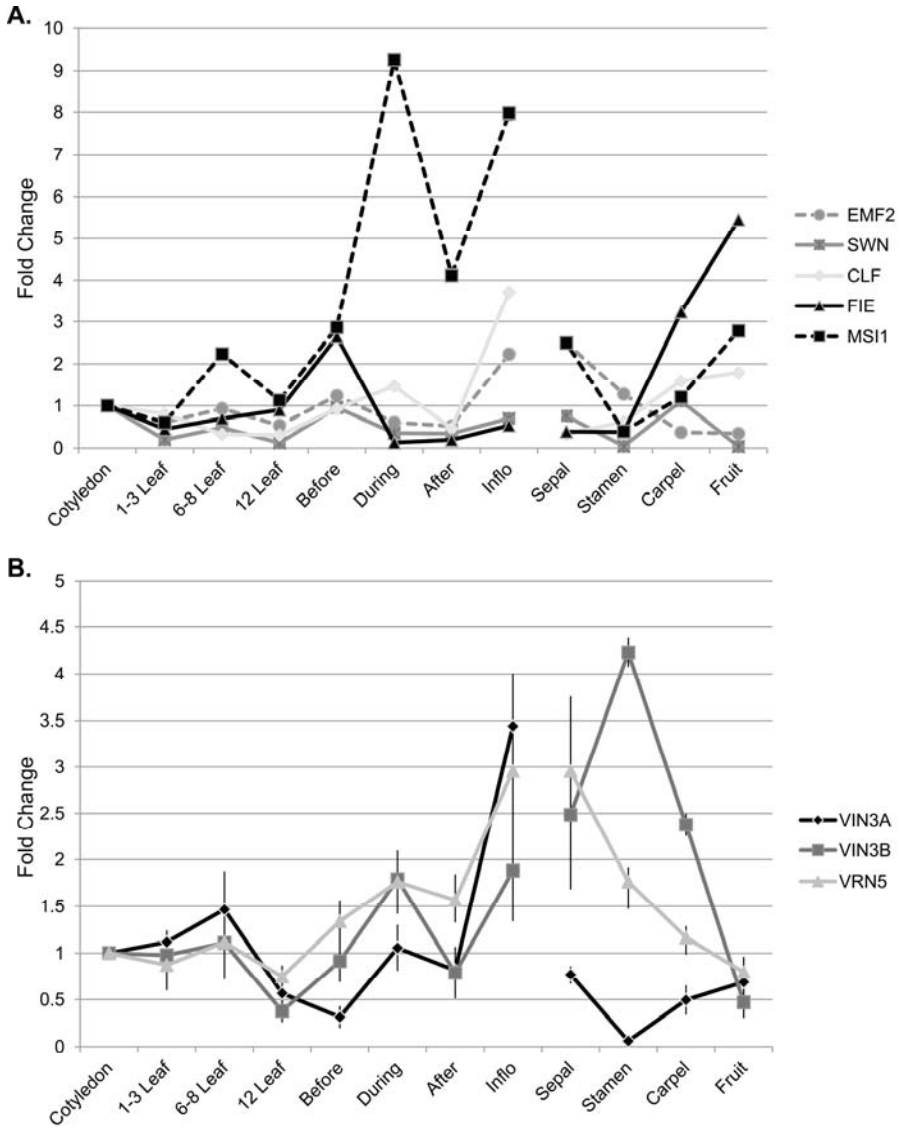


Figure 2.6: qRT-PCR analysis of expression of the PRC2 and VEL PHD genes in *A. vulgaris*. Tissue from 3-10 plants was collected and pooled at each stage. For each data point, three technical replicates were analyzed. *AqIPP2* expression was used for normalization. **A.** Average fold change of the PRC2 complex genes throughout the life cycle of *A. vulgaris* normalized to the cotyledon stage sample with SD error bars. **B.** Average fold change of the VEL PHD family throughout the life cycle of *A. vulgaris* normalized to the cotyledon stage sample with SD error bars.

Parental Expression of *AqCLF* and *AqSWN* in *Aquilegia* Endosperm

As discussed above, select members of the PRC2 complex in several model species exhibit parent-of-origin-specific patterns of imprinting and, hence, expression patterns in the endosperm. We therefore sought to determine the imprinting patterns of specific PRC2 homologs in *Aquliegia*.

We chose to focus on the E(z) homologs *AqCLF* and *AqSWN* because in almost every case where PRC2 gene imprinting has been described, the targeted genes are one of several copies present in the genome, including the *SWN* paralog *MEA* in *A. thaliana* and *SWN* homolog *Mez1* maize, respectively (Haun et al. 2007; Spillane et al. 2007; Rodrigues et al. 2010). Our experiment took advantage of the fact that many *Aquilegia* species are interfertile and their seeds have large, persistent endosperm (Fig. 2.7A) (Prazmo 1965). Genetic variation between *Aquilegia* species is low and what variation exists is not fixed (Hodges and Arnold 1994). Therefore we tested several plants and identified one *Aquilegia vulgaris* and one *Aquilegia canadensis* plant bearing polymorphisms in the 3'UTRs of *AqCLF* and *AqSWN* that could be distinguished by restriction digestion (Fig. 2.7B and C). We then conducted reciprocal crosses between the relevant individuals and collected the hybrid seeds. The seeds were bisected perpendicular to the micropyle to separate the endosperm from the embryo (Fig. 2.7A) and cDNA libraries were made from each half. We then amplified and digested the relevant 3' UTR fragments of *AqCLF* and *AqSWN* from both halves of the seeds as well as leaf tissue from both parents. We found no evidence for imprinting in either of these loci (Fig. 2.7B and C). While the parental alleles could be easily distinguished by

restriction digest, the gene segments purified from the hybrid endosperm clearly contained both polymorphisms. While the *A. canadensis* allele of *AqSWN* appears to be present at a lower level than that of *A. vulgaris* in the endosperm sample from cross 1 (*A. canadensis* female x *A. vulgaris* male), the alleles appear to be present at approximately equal levels in the endosperm sample from cross 2 (*A. vulgaris* female x *A. canadensis* male). This difference seems to be due to a stochastic difference in allele amplification since other duplicate reactions appeared more equivalent, but we chose to show one entire set of concurrent reactions.

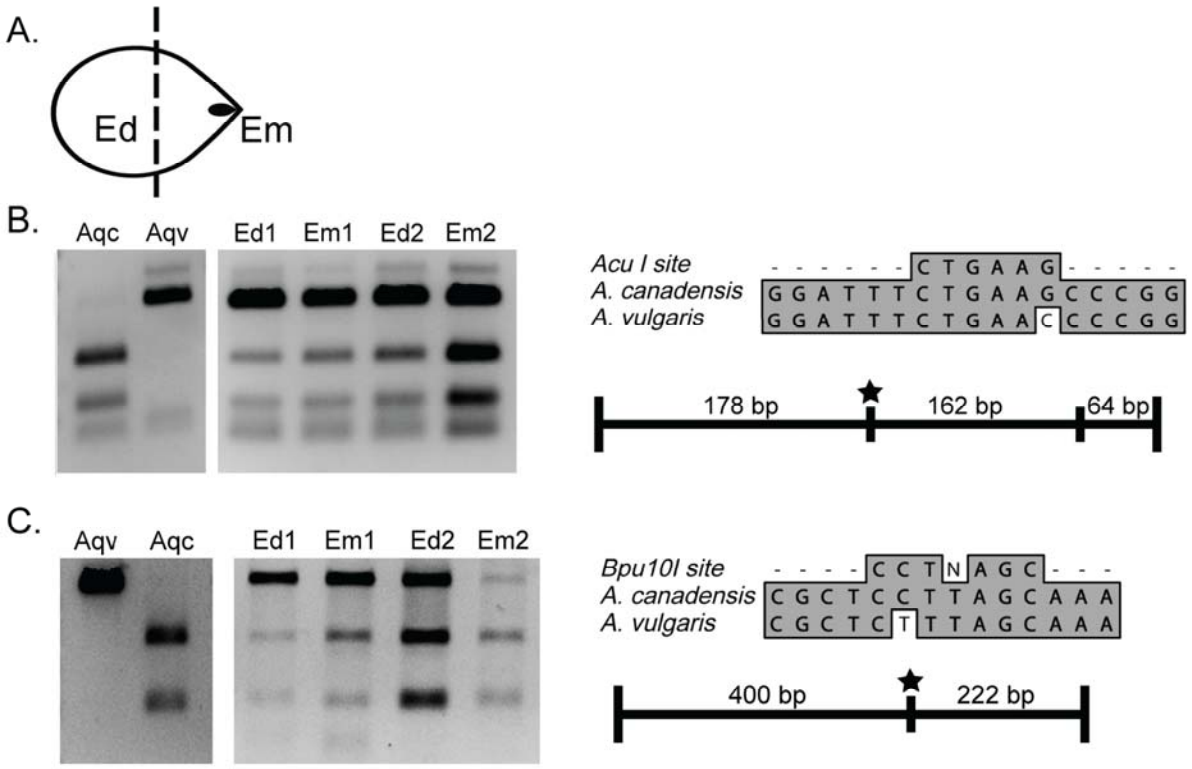


Figure 2.7: *AqCLF* and *AqSWN* are not imprinted in *Aquilegia* endosperm. **A**. Diagram indicating position of embryo within the *Aquilegia* seed. Dotted line indicates orientation of seed bisection. **B.** Acu I digests of the 3' UTR of *AqCLF* purified from parental controls and hybrid seeds (both the endosperm (Ed) and embryo (Em) halves) run on a 2% agarose gel stained

Figure 2.7 Continued: with ethidium bromide. Acu I cuts *Aquilegia canadensis* (Aqc) twice but only cuts *Aquilegia vulgaris* (Aqv) once. Hybrid seeds from reciprocal crosses (cross Em1/Ed1: *A.canadensis* female x *A. vulgaris* male, cross Em2/Ed2: *A. vulgaris* female x *A. canadensis* male) contain both polymorphisms. **C**. Bpu10I digests of the 3' UTR of *AqSWN* purified from parental controls and hybrid seeds (both the endosperm (Ed) and embryo (Em) halves) run on a 2% agarose gel stained with ethidium bromide. Bpu10I cuts *Aquilegia canadensis* (Aqc) once but does not cut *Aquilegia vulgaris* (Aqv). Hybrid seeds from reciprocal crosses (cross Em1/Ed1: *A. canadensis* female x *A. vulgaris* male, cross Em2/Ed2: *A. vulgaris* female x *A. canadensis* male) contain both polymorphisms.

These findings do not mean that imprinting of other loci does not play a role in endosperm development in *Aquilegia*, on the contrary, it seems very likely that it does (Baroux et al. 2002). What they may suggest, however, is that the imprinting observed with *A. thaliana MEA* and *FIS2*, as well as the grass loci *Mez1*, *ZmFIE1*, and *OsFIE1*, is related to the subfunctionalization of particular PRC2 paralogs for a role in endosperm development. In the cases of *MEA* and *FIS2*, this specialization is further associated with a higher rate of molecular evolution as indicated by statistical tests or exceedingly long-branch lengths (Spillane et al. 2007; Chen et al. 2009). Of course, this is not exclusively the rule as the single copy *A. thaliana* locus *FIE* also shows imprinting (Ohad et al. 1996). Unfortunately, we were not able to identify suitable polymorphisms in *AqFIE* but, hopefully, such tests will be feasible in the future.

2.4 Conclusions

- *A. coerulea* has a simple complement of PRC2 homologs with no recent duplications
- The PRC2 genes are broadly expressed throughout *A. vulgaris* development with no obvious tissue or stage specialization
- The ancient paralogs, *AqCLF* and *AqSWN,* do not appear to be imprinted in *Aquilegia* endosperm.
- *A. coerulea* has four members of the VEL PHD family, three of which are similar to *A. thaliana* genes known to function in flowering time.
- VEL PHD gene expression in *A. vulgaris* is not confined to vernalization as seen with *VIN3* in *A. thaliana,* but is moderately increased both during vernalization and in the inflorescence.
- We have now identified a set of chromatin remodeling gene homologs in *Aquilegia* for further functional studies as well as phylogenetic analyses.

Chapter 3:

Conserved roles for Polycomb Repressive Complex 2 in the regulation of lateral organ development in *Aquilegia x coerulea* ‘Origami’.

3.1: Introduction

Maintenance of proper gene expression in differentiated cells is essential for the development of multicellular organisms. Epigenetic regulation is one mechanism used to maintain gene expression (Reviewed in: Holliday 1994; Russo et al. 1996; Feil 2008). One family of proteins with deeply conserved functions in epigenetic regulation is the Polycomb Group (PcG). The PcG was first discovered in *Drosophila melanogaster* as repressors of the HOX genes (Lewis 1978). Several PcG complexes exist in both plants and animals, each with distinct functions in epigenetic silencing (Reviewed in: Hennig and Derkacheva 2009; Sawarkar and Paro 2010). However, only the Polycomb Repressive Complex 2 (PRC2) has been well characterized in plants (Reviewed in: Hennig and Derkacheva 2009; Köhler and Hennig 2010). The main function of the PRC2 complex is trimethylation of lysine 27 of histone H3 (H3K27), a histone modification known to suppress gene expression (Schubert et al. 2006). The PRC2 contains four core proteins; the histone methyltransferase *Enhancer of Zeste* (*E(z)),* and three other proteins thought to enhance PRC2 binding to nucleosome (Nekrasov et al. 2005): *Suppressor of Zeste 12 (Su(z)12)* and *Extra Sex Combs (ESC),* known as *EMBRYONIC FLOWER 2* (*EMF2*) and *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), respectively, in plants, and *Multi-Copy Suppressor of IRA 1 (MSI1*) (Reviewed in: Pien and Grossniklaus 2007). The *E(z)* lineage in plants underwent an ancient duplication such that most angiosperms have at least two paralogs, known as *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*) (Spillane et al. 2007). Many plant species have additional duplications in the core PRC2 loci that allow them to form several PRC2 complexes often with distinct functions (Whitcomb et al. 2007; Luo et al. 2009).

PRC2 is involved in a number of important developmental transitions. In the plant model system *A. thaliana*, these functions include endosperm development, early repression of flowering to allow proper vegetative development, the eventual transition to flowering, and flower organogenesis (Goodrich et al. 1997; Gendall et al. 2001; Yoshida et al. 2001; Kohler et al. 2003b). In grasses, the PRC2 complex may play a role in floral induction (rice and barley), flower development (rice), suppressing cell divisions in the unfertilized ovule (rice), and endosperm development (rice and maize) (Luo et al. 2009; Oliver et al. 2009; Rodrigues et al. 2010). In the moss model *Physcomitrella patens*, PRC2-dependent remodeling may be required for the switch from gametophyte to sporophyte development (Mosquna et al. 2009; Okano et al. 2009).

In addition to its role in developmental transitions, PRC2 has been suggested to play major roles in lateral organ development in *A. thaliana*. In fact, the first description of a plant PRC2 function was discovered with the characterization of the *clf* mutant in *A. thaliana* (Goodrich et al. 1997). The *clf* plants had severely curled leaves, smaller narrower sepals and petals, and partial homeotic transformations of sepals and petals towards carpel and stamen identity. Two MADS box genes, the C class member *AGAMOUS* (*AG)* and the B class representative *APETALA3* (*AP3*) were shown to be over-expressed in *clf* mutants, suggesting that the PRC2 complex was required for stable repression of these genes (Goodrich et al. 1997). This was particularly interesting because MADS box genes regulate homeotic floral organ identity in plants somewhat analogously to the way HOX genes regulate segment identity in animals (Bowman et al.

1989; Bowman et al. 1991; Foronda et al. 2009; Bowman et al. 2012). Further studies have subsequently shown that the E class MADS *SEPALLATA3* (*SEP3*) is similarly up-regulated in *clf* mutants (Lopez-Vernaza et al. 2012). PRC2 has also been shown to regulate the expression of the class I KNOX genes during vegetative development. The class I KNOX genes are a family of homeobox domain containing loci in plants that have conserved roles in promoting pluripotency in the shoot apical meristem and in compound leaf development (Bharathan et al. 2002; Wagner 2003). Katz et al (2004) found that in addition to the phenotypes reported in *clf* mutant plants, *FIE* cosuppressed plants also had loss of apical dominance and fasciated stems, rolled leaves with varying degrees of serration, loss of phyllotaxy in the inflorescence, and many problems with ovary and ovule development (Katz et al. 2004). They further demonstrated that several class I KNOX genes, including *BREVIPEDICELLUS* (*BP*), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*), and *SHOOTMERISTEMLESS* (*STM*), were over-expressed in rosette leaves of *FIE* silenced plants. In *clf* mutants, *STM* and *KNAT2* were over-expressed but *BP* was not, possibly because its paralog *SWN* was acting redundantly (Katz et al. 2004). The class I KNOX genes *MOSS KNOTTED1-LIKE 2* and *5* (*MKN2* and *MKN5*) were also shown to be over-expressed in *PpFIE* mutant gametophytes (Singer and Ashton 2007; Mosquna et al. 2009), suggesting that PRC2 targeting of the class I KNOX genes may be conserved.

While the functions of the floral ABC class and type I KNOX genes are thought to be deeply conserved, comparative studies of their regulation have largely focused on upstream transcription factors, such as *LEAFY* or ARP family members (Kim et al.

2003a; Maizel et al. 2005). In order to begin addressing the question of whether PRC2-targeting interactions are similarly conserved, we have examined the functions of PRC2 members in lateral organ development in the emerging model system *Aquilegia*. The genus *Aquilegia* is a member of an early diverging lineage of the eudicotyledonous flowering plants, the Ranunculales, that arose before the radiation of the core eudicots (Reviewed in: Hodges and Kramer 2007). It therefore can be used as a rough phylogenetic midpoint between *A. thaliana* and model systems in the grasses (Kramer and Hodges 2010). Additionally, many ecological, evolutionary and genetic studies have been conducted in *Aquilegia* over the past 50 years and it has a small genome (n=7, approximately 300 Mbp) with a number of tools, including the fully sequenced *Aquilegia x coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) (Reviewed in: Hodges and Kramer 2007; Kramer 2009). The reverse genetic tool virus-induced gene silencing (VIGS) has been optimized in several species of *Aquilegia* (Gould and Kramer 2007) for both leaf and floral development (Kramer et al. 2007; Sharma et al. 2011; Pabón-Mora et al. 2013; Sharma and Kramer 2013). Previously we examined the evolution and expression of the PRC2 family in *Aquilegia* (Gleason and Kramer 2012) and found that the genome contains a simple complement of PRC2 homologs: one copy each of the two plant *E(z)* homologs, *AqCLF* and *AqSWN*; an *ESC* homolog, *AqFIE*; a *Su(z)12* homolog, *AqEMF2*; and a copy of *MSI1, AqMSI1*. We initially assessed gene expression throughout *Aquilegia vulgaris* development due to its strong vernalization dependency and found no obvious tissue or stage specialization. Furthermore, the ancient paralogs, *AqCLF* and *AqSWN,* are not imprinted in *Aquilegia*

endosperm as is seen in other plant species (Rodrigues et al. 2010; Gleason and Kramer 2012).

In the current study we have used VIGS to knock down the expression of *AqFIE* and *AqEMF2* in unvernalized and vernalized *Aquilegia x coerulea* 'Origami' plants using the *ANTHOCYANIN SYNTHASE* (*AqANS*) as a marker gene. We find that PRC2 plays a role in leaf and floral organ development in *A. x coerulea*, particularly via down-regulation of the floral MADS box genes. This has allowed us to identify PRC2 targets that appear to be conserved between *A. thaliana* and *Aquilegia* as well as some novel PRC2-regulated pathways.

3.2: Methods

Virus-Induced Gene Silencing

The *Aquilegia* VIGS protocol was preformed as described previously (Gould and Kramer 2007). TRV2-*AqCLF-AqANS,* TRV2-*AqSWN-AqANS,* TRV2-*AqFIE-AqANS* and TRV2-*AqEMF2-AqANS* constructs were prepared by PCR amplifying approximately 300 bp regions of each gene using primers that added *Eco*R1 and XbaI restriction sites to the 5' and 3' ends of the PRC products (see Appendix 1). The PCR products were then purified and cloned into the TRV2-*AqANS* construct (Gould and Kramer 2007) and electroporated into *Agrobacterium* strain GV101. *A. x coerulea* seedlings were grown to approximately the 4 to 6 leaf stage and then either treated as described in Gould and Kramer (2007) for unvernalized samples or as described in Sharma and Kramer (2013) for plants that had been vernalized for approximately 4 weeks at 4^{o}C (Gould and

Kramer 2007; Sharma and Kramer 2013). Leaves, petals, and sepals showing *AqANS* silencing were photographed, collected, and stored at -80^{o}C for RNA analysis.

RT-PCR

RNA was extracted from control (*AqANS* silenced*)* and experimental (*AqFIE* and *AqEMF2* VIGS treated) tissue. For leaves, the RNeasy Mini Kit (Qiagen, Valencia, CA) was used. For petals and sepals RNA was extracted using the Pure-Link Plant RNA Reagent small scale RNA isolation protocol (Ambion, Austin, TX). RNA was treated with Turbo DNase (Ambion, Austin, TX) and cDNA was synthesized from 1 µg of total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT) primers. cDNA was diluted 1:5 prior to use.

Amplification was performed using AccuStart PCR SuperMix (Quanta Biosciences Inc, Gaithersburg, MD). The amplification program began with 1 minute activation step at 94^{o}C, followed by a 20 second denaturing step at 94^{o}C, a 15 second annealing step at 55^{o}C, and a 15 second extension at 72^{o}C, repeated for 30 cycles. This cycle number was chosen for optimal detection of *AqFIE* and *AqEMF2*, which are expressed at relatively low levels in mature organs, especially compared to the high expression levels of *AqIPP2*. All primers used are listed in Appendix 1. Amplification of *ISOPENTYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE2 (AqIPP2)* was used as a positive control (Ballerini and Kramer 2011; Sharma et al. 2011).. To test for expression of *APETALA3-1 (AqAP3-1), APETALA3-2 (AqAP3-2), APETALA3-3 (AqAP3-3),* and *FUL-like- 1 (AqFL1*) in VIGS treated leaves, cDNA from several leaves

were pooled together prior to amplification. The control pool consisted of control leaves 1-4, the *AqFIE* VIGS treated pool consisted of *AqFIE* leaves 3-6, and the *AqEMF2* VIGS treated pool consisted of *AqEMF2* leaves 1-4.

qRT-PCR

cDNA was prepared from VIGS treated tissue as described above. For the carpel sample, carpels were collected from 3 anthesis stage wild type plants and pooled together. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and treated as described above. cDNA from VIGS treated tissue was then pooled together and diluted 1:10. The control sepal pool consisted of control sepals 1-4, the control petal pool consisted of control petals 1-4, the *AqFIE* sepal pool consisted of *AqFIE* VIGS treated sepals 2, 3, 5, and 6, the *AqFIE* petal pool consisted of *AqFIE* VIGS treated petals 2, 3, 5, and 6, the *AqEMF2* sepal pool consisted of *AqEMF2* VIGS treated sepals 2, 3, and 4s, and the *AqEMF2* petal pool consisted of *AqEMF2* VIGS treated petals 1 and 2. qRT-PCR was performed using PerfeCTa qPCR FastMix, Low ROX (Quant Biosciences Inc., Gaithersburg, MD) in the Stratagene Mx3005P QPCR system to study the relative expression of *AqAG1* and *AqAG2. AqIPP2* expression was used for value normalization. All primers are listed in Appendix 1.

Microscopy

Petals from wild type, *AqANS* VIGS treated, and *AqEMF2* VIGS treated plants were stored at -80^{o}C and then warmed to room temperature and mounted whole on glass slides in water. Cells were visualized in the Harvard Center for Biological Imaging on a

Zeiss AxioImager Z2 microscope using trans-illumination with white light. Images were taken using a Zeiss AxioCam Mrc digital camera.

3.3: Results

We treated both unvernalized and vernalized plants with TRV2 constructs containing either *AqANS-AqFIE* or *AqANS-AqEMF2* fragments. Phenotypes of *AqFIE* and *AqEMF2* silenced plants were equivalent and will be discussed together. We also treated a small number of unvernalized plants with *AqANS-AqCLF* and *AqANS-AqSWN* VIGS constructs. Phenotypes from these plants were similar to those seen in *AqFIE* and *AqEMF2,* but were weaker (data not shown), most likely due to partial redundancy between *AqCLF* and *AqSWN.* Thus we chose to focus on *AqFIE* and *AqEMF2* VIGS treated tissue. As is common for VIGS-treated plants, we recovered a range of phenotypes (Gould and Kramer 2007). In the current experiment there is the added component that phenotypes are likely due to mis-expression of PRC2 target genes, and are therefore likely to have an added complexity due to variable ectopic expression of a potentially wide range of target loci.

Vegetative Phenotypes

Wild type *A. x coerulea* leaves are compound, typically bearing three leaflets that are themselves divided into two to three lobes (Fig. 3.1A). Although these leaflets are often relatively deeply lobed, they do not generally produce elongated, higher order petiolules. However, *A. x coerulea* does display heteroblasty over the course of its lifespan (see Appendix 3). In late reproductive adult stages, higher order petiolules may

be observed in which the central lobe of each leaflet becomes itself a separate leaflet borne on a petiolule (see Appendix 3). Using the terminology of Kim et al. (Kim et al. 2003a), all of these leaf forms are non-peltately palmate in that the leaflets are not radially positioned around the terminus of the rachis.

The leaves of *AqFIE* and *AqEMF2* silenced plants showed a complex set of phenotypes. The most consistently observed perturbation was curled or ruffled laminae that typically curled toward the abaxial surface (Fig. 3.1F, H, J-L). We also observed an increased frequency of higher order branching in which fully formed petiolules developed within the leaflet, creating as many as ten or twelve distinct leaflets rather than the usual three (Fig. 3.1B-F, H, L, see also Appendix 3). When quantified (see Appendix 3), this increase in branching is significant at $p<0.05$ for unvernalized lateral leaflets but not significant for the other stages/leaflet types. However, it is obvious that there is much more variation in silenced leaflets than in controls. In some cases, the margins of the laminae had additional lobing relative to control leaves (Fig. 3.1B-E, K) and, in a small number of cases, the central lobe of the terminal leaflet was severely reduced (Fig. 3.1D, M). Laminar area was highly variable with some leaflets appearing to have expanded area (Fig. 3.1F) while others seemed reduced (Fig. 3.1G, I, M). Rarely, ectopic finger-like projections were observed on the adaxial surface of lamina (Fig. 3.1F), which was never observed in control leaflets.

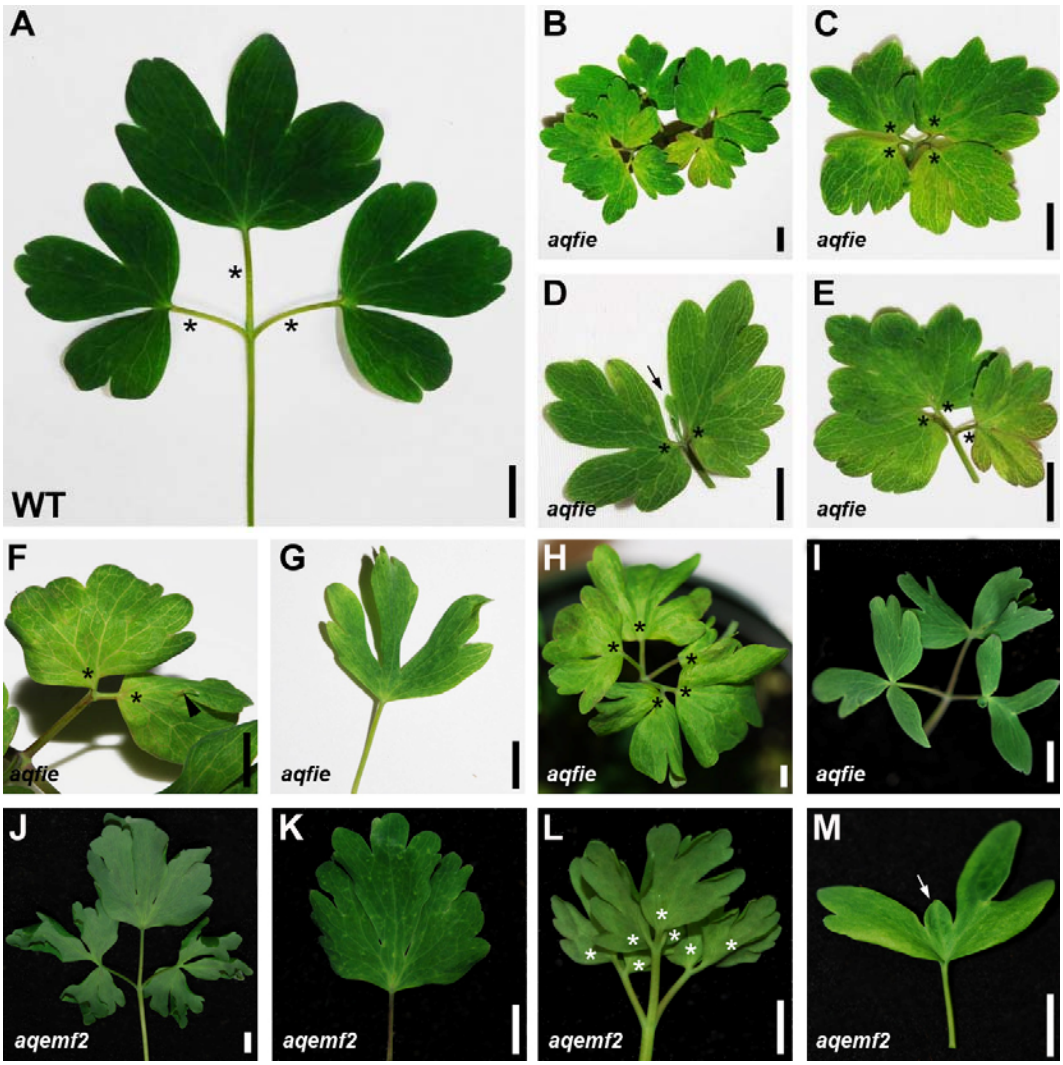


Figure 3.1: Vegetative phenotypes of PRC2 VIGS treated plants. **A.** *AqANS*-treated leaf (termed WT) with three lobed leaflets. Petiolules are marked with asterisks. **B-I.** *AqFIE*-silenced leaves and leaflets (abbreviated *aqfie*). **B.** Entire leaf with highly branched leaflets. **C-E.** Each leaflet from the leaf shown in B with petiolules marked with asterisks and reduced central lobe indicated with an arrow. Leaflets are arranged in clockwise order starting with the left lateral leaflet in B. **F.** Leaflet with curled laminae, increased branching (asterisks) and ectopic outgrowth on the adaxial lamina (white arrowhead). **G.** Leaflet with reduced lamina and narrow lobes that are deeply divided.**H.** Entire leaf showing increasing internal branching (asterisks) and curling. **I.** Entire leaf with deep lobes and aberrantly shaped laminae.

Figure 3.1 Continued: J-M. *AqEMF2*-silenced leaves (abbreviated *aqemf2*). **J.** Entire leaf showing curled/ruffled laminae and deep lobing. **K.** Central leaflet from J exhibiting curled laminae, increased degree of lobing and serration. **L.** Entire leaf with internal branching (asterisks) and curled laminae. **M.** Leaflet with reduced central lobe (arrow). Scale bars: 1 cm.

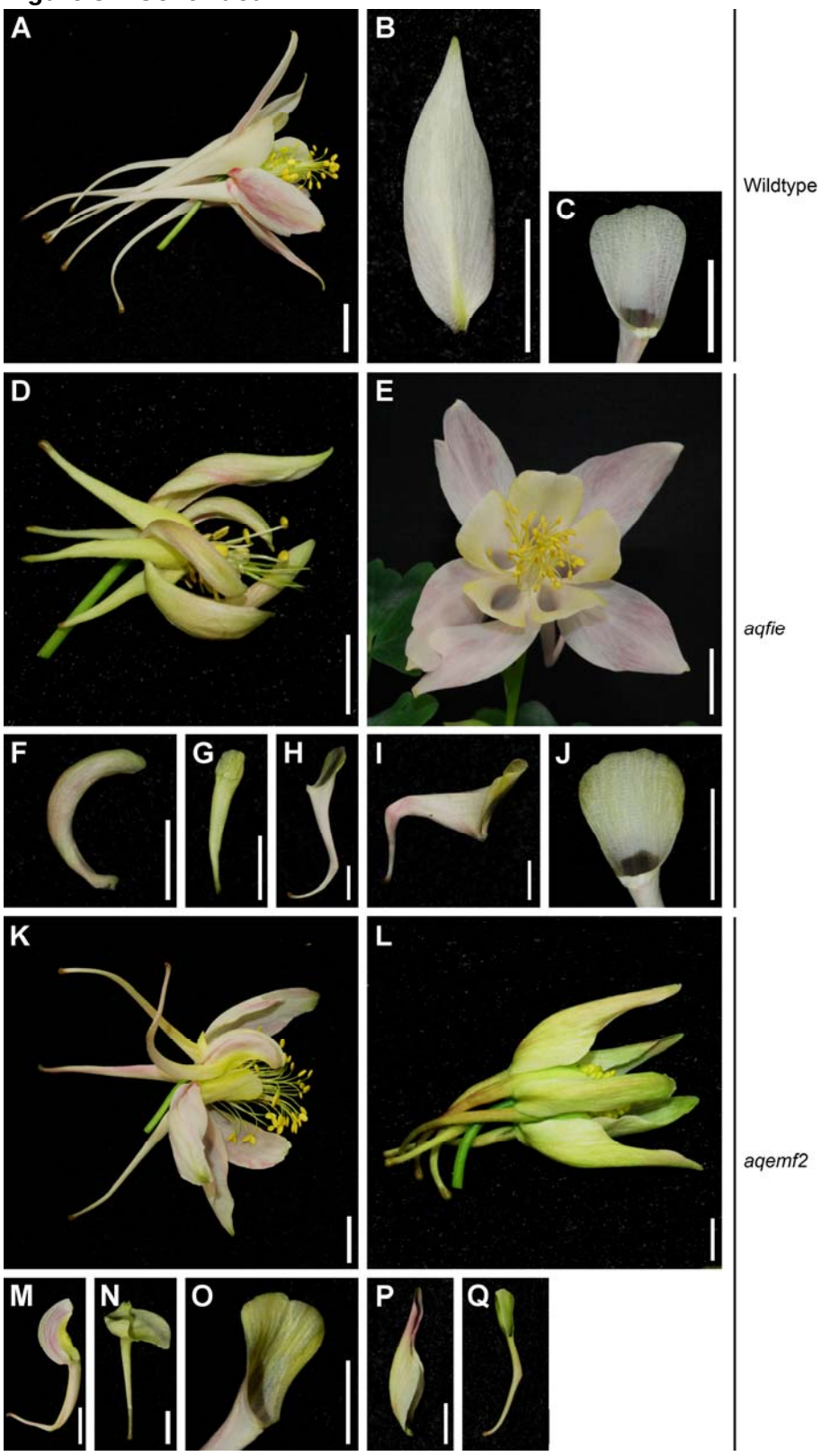
Floral Phenotypes

Wild type *A. x coerulea* flowers possess five organ types: sepals, petals, stamens, staminodia and carpels (Kramer et al. 2007). We have focused on the sepals and petals because they showed strong phenotypes in the silenced flowers. Wild type sepals are flat and ovate with an entire margin (Fig. 3.2A-B). The petals are notable for the presence of a long hollow nectar spur, which forms near the attachment point (Fig. 3.2A). This feature divides the organ into two regions, the proximal spur and the distal limb. Spurs are typically 5-6 cm in length and slightly curved while the limb region is relatively flat with a rounded, weakly lobed margin (Fig. 3.2C). Sepals in *AqFIE* and *AqEMF2* silenced plants were commonly narrower than wildtype organs and dramatically folded towards the adaxial surface (Fig. 3.2D, F, L, P). Petals were often narrowed and stunted in severely affected flowers (Fig. 3.2D, G, Q) or exhibited sharply bent spurs (Fig. 3.2H-I, K, M, Q). In several *AqEMF2*-silenced flowers, the sepals exhibited chimeric petal identity including ectopic spur formation (Fig. 3.2M-N). Perhaps most surprising, many of the perianth organs had a definite yellow hue, with the petal limbs showing particularly intense yellow coloration (Fig. 3.2E, I-M, O). Such coloration was not observed in *AqANS*-silenced control flowers (Fig. 3.2A-C). Examination of the *AqFIE*- and *AqEMF2*-silenced organs under high magnification reveals that yellow

pigment is deposited in plastids (Fig. 3.3A), consistent with carotenoids rather than the vacuole-based aurones that are produced in some *Aquilegia* species (Vishnevetsky et al. 1999; Ono et al. 2006).

Figure 3.2: Floral phenotypes of PRC2 VIGS treated plants. **A-C.** *AqANS*-silenced control flower and perianth organs (termed Wildtype). **A.** Entire flower. **B.** Entire sepal. **C.** Petal limb. **D-J.** *AqFIE*-silenced flowers and organs (abbreviated *aqfie*). **D.** Severely affected flower. **E.** Moderately affected flower. **F.** Narrow, folded sepal of flower in D. **G.** Narrow, stunted petal of flower in D. **H-I.** Petals with bent spurs from moderately affected flowers. **J.** Yellow limb of moderately affected petal. **K-Q.** *AqEMF2*-silenced flowers and organs (abbreviated *aqemf2*). **K-L.** Severely affected flowers. **M-N.** Sepal/petal chimeras from first whorl of flowers such as K. **O.** Yellow limb of second whorl petal from flower in K. **P.** Narrow, folded sepal from flower in L. **Q.** Narrow, bent petal from flower in L. Scale bars: 1 cm.

Figure 3.2 Continued:



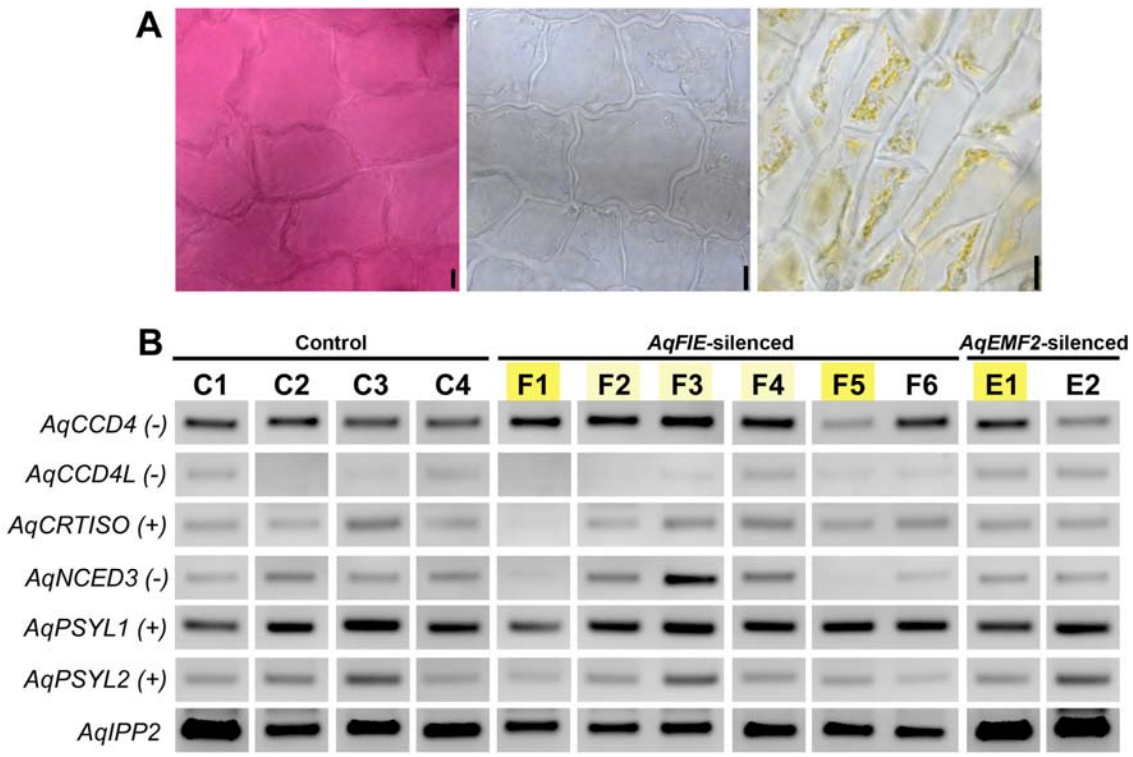


Figure 3.3: The PRC2 regulates carotenoid production in *A. coerulea* petals. **A.** High magnification views of epidermal cells in *A. x coerulea* petal limbs. From left to right: Anthocyanin of untreated petal limb (anthocyanin is deposited in the vacuole, resulting in a very even distribution of color), almost complete lack of color in *AqANS*-silenced petal limb, and punctate pattern of carotenoid deposition in plastids of *AqEMF2*-silenced petal limb. **B.** Expression of several *A. x coerulea* homologs of genes important in carotenoid production (*CRTISO* and *PSY*) and degradation (*CCD4* and *NCED3*) in *AqANS*-silenced control petals (C1-C4) and *AqFIE* (F1-F6) and *AqEMF2* (E1 and E2) treated petals. Petals with strong yellow pigment are highlighted in dark yellow (F1, F5, and E1) and petals with pale yellow pigment are highlighted in light yellow (F2-F4). The expression of these genes is not consistently affected in the *AqFIE* and *AqEMF2* silenced petal samples. It is possible that other genes in the carotenoid pathway are being misexpressed. Scale bars: 10 μm.

Assessment of *AqFIE* and *AqEMF2* down-regulation

Due to limited RNA availability, we used standard RT-PCR to assess target gene down-regulation in leaves, sepals and petals. Even in control tissue, *AqFIE* and *AqEMF2* are expressed at low levels relative to the loading control *AqIPP2.* This analysis demonstrated that in the TRV2-*AqFIE- AqANS* treated plants, *AqFIE* was strongly down-regulated, being undetectable in a number of samples (Fig. 3.4A and Fig. 3.5). Likewise, *AqEMF2* expression is reduced to undetectable levels in most tested *AqEMF2*-silenced samples (Fig. 3.4A and Fig. 3.5). We also tested for *AqEMF2* in *AqFIE*-treated plants and vice versa, and found that *AqEMF2* levels are often reduced in *AqFIE*-treated leaves, although the reciprocal is generally not true (Fig. 3.4A). Furthermore, we tested the other PRC2-complex members, *AqCLF* and *AqSWN*, and found no consistent evidence of their down-regulation in either type of silenced tissue (Fig. 3.6A).

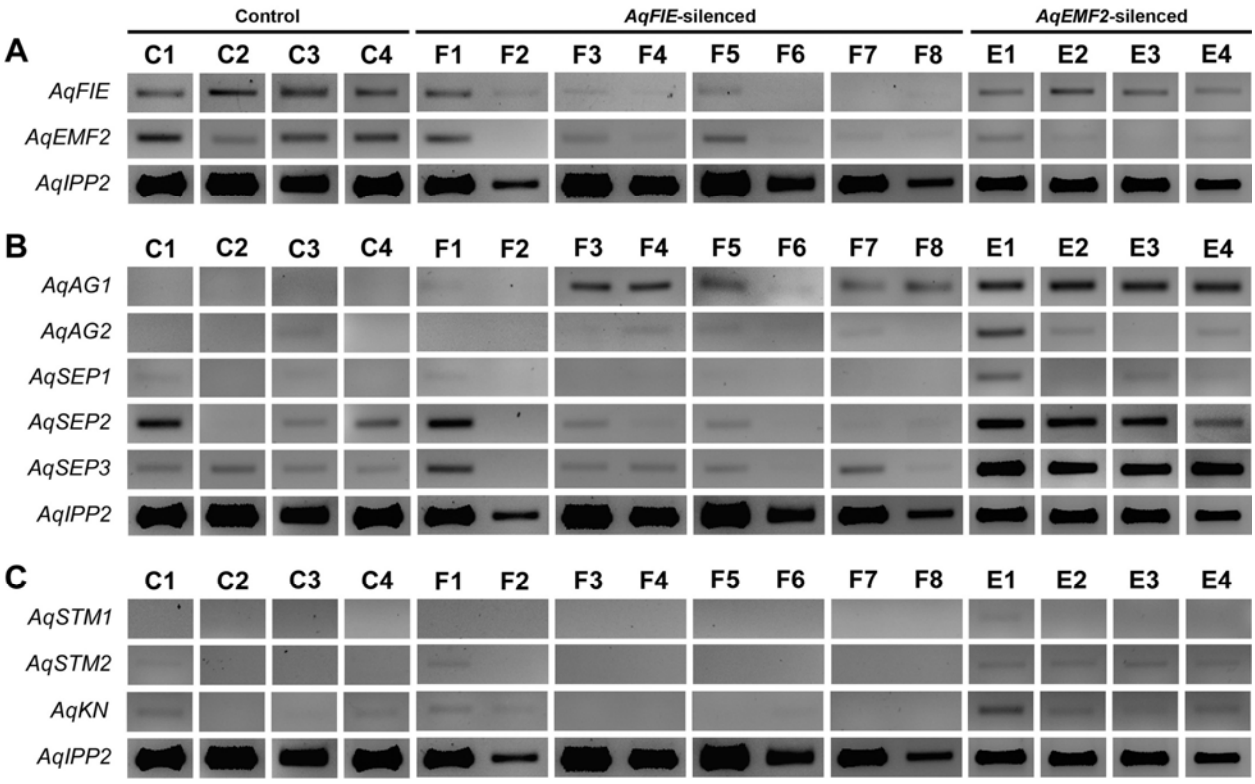


Figure 3.4: RT-PCR expression data in PRC2 VIGS treated leaves. *AqIPP2* was used as a loading control. The expression of *AqFIE* and *AqEMF2* are low relative to the expression of *AqIPP2*. **A.** Expression of *AqFIE* and *AqEMF2* in *AqANS*-silenced control leaves (C1-C4), and *AqFIE* (F1-F8) and *AqEMF3* (E1-E4). *AqFIE* is clearly down-regulated in *AqFIE*-silenced tissue and, likewise, *AqEMF2* is down-regulated in *AqEMF2*-silenced tissue. Interestingly, *AqEMF2* also appears to be down-regulated in *AqFIE*-treated leaves but *AqFIE* expression is unaffected in *AqEMF2*-treated leaves. **B.** Expression of several floral organ identity genes in *AqANS*-silenced control leaves (C1-C4), and *AqFIE* (F1-F8) and *AqEMF3* (E1-E4). In several of the *AqFIE* down-regulated leaves and all of the *AqEMF2* down-regulated leaves, *AqAG1* is over-expressed compared to *AqANS*-silenced control leaves. While the expression of the *SEPALLATA* homologs is variable in both control and experimental leaves, *AqSEP3* may be up-regulated in some of the *AqFIE*- and all of the *AqEMF2-silenced* leaves.

Figure 3.4 Continued: C. Expression of several of the *A. x coerulea* class I KNOX genes in *AqANS*-silenced control leaves (C1-C4), and *AqFIE* (F1-F8) and *AqEMF3* (E1-E4). Expression of these genes is unaffected in the mature *AqFIE*- and *AqEMF2*-silenced leaves.

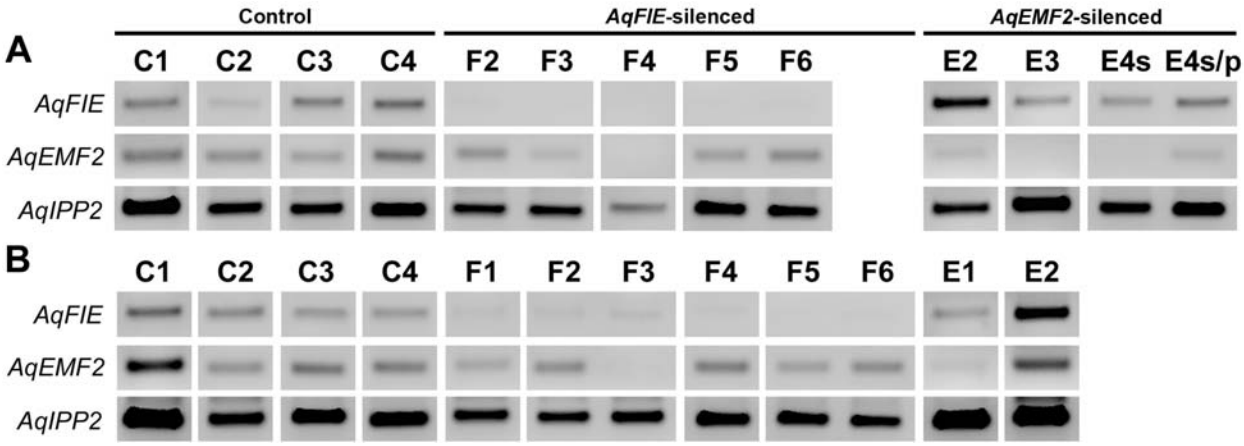


Figure 3.5: Expression of *AqFIE* and *AqEMF2* in VIGS treated floral organs. **A.** Expression of *AqFIE* and *AqEMF2* in *AqANS*-silenced control sepals (C1-C4), and *AqFIE*- (F2-F6) and *AqEMF3*- (E1-E4s/p) treated first whorl organs. *AqFIE* is down-regulated in all of the *AqFIE*-treated sepals. Likewise, *AqEMF2* is down-regulated in *AqEMF2*-treated first whorl organs. Unlike the pattern in leaves, *AqEMF2* is not down-regulated in *AqFIE*-treated sepals. **B.** Expression of *AqFIE* and *AqEMF2* in *AqANS*-silenced control petals (C1-C4), and *AqFIE*- (F1-F6) and *AqEMF2*- (E1 and E2) treated petals. *AqFIE* is down-regulated in all of the *AqFIE*-treated petals while *AqEMF2* is down-regulated in E1 and also in F3.

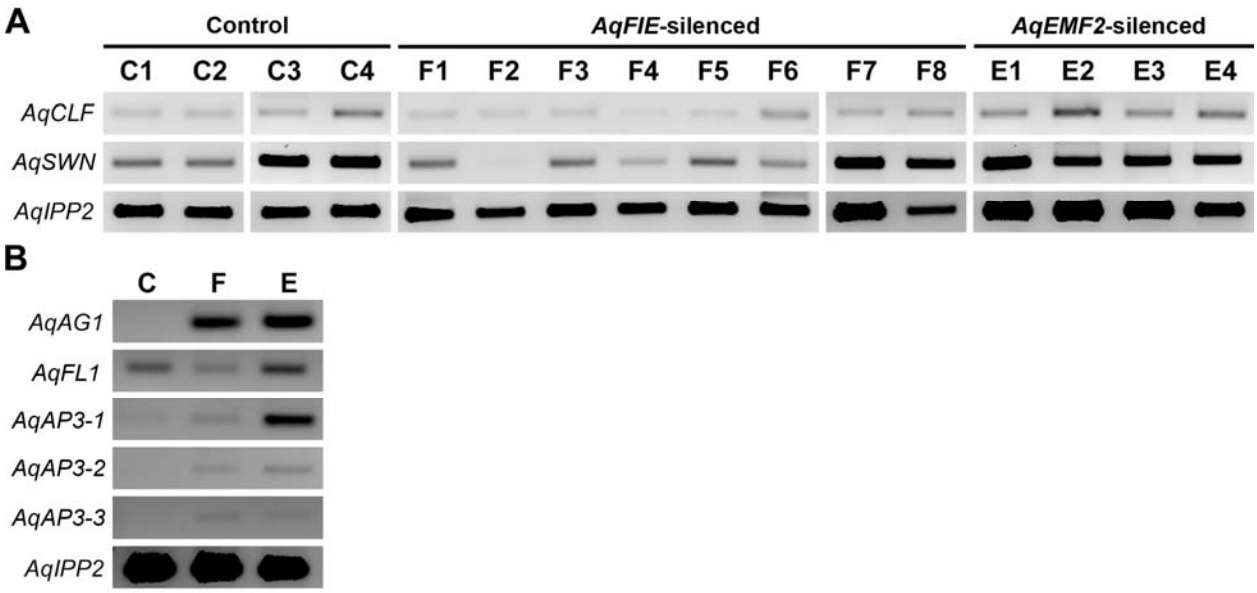


Figure 3.6: Additional candidate gene expression in PRC2 VIGS treated leaves. **A.** Expression of *AqCLF* and *AqSWN* in *AqFIE*- and *AqEMF2*-treated leaves. Although *AqEMF2* appears to be down-regulated in some *AqFIE*-silenced leaves, the expression of *AqCLF* and *AqSWN* in these leaves is not affected. **B.** Expression of *AqAG1*, *AqFL1, AqAP3-1, AqAP3-2,* and *AqAP3-3* in pooled *AqANS* silenced control leaves (C) and *AqFIE* (F) and *AqEMF2* (E) silenced leaves. *AqAP3-1 AqAP3-2* and *AqAP3-3* is moderately up-regulated in both *AqFIE* and *AqEMF2* silenced tissue while *AgFL1* expression is unaffected.

Assessment of Candidate Gene Expression

We tested for ectopic expression of a wide panel of potential target genes, with a focus on the floral organ identity loci and type I KNOX homologs (Fig. 3.4, Fig. 3.7, and Fig. 3.6B). One of the two *A. x coerulea* the C class MADS box genes, *AGAMOUS 1* (*AqAG1*) (see Appendix 2 for all gene identification numbers), is consistently up-regulated in silenced leaves and floral organs. The second *AGAMOUS* homolog, *AGAMOUS2* (*AqAG2*), may also be slightly up-regulated in some of the leaves,

although *AqAG2* shows basal expression in control floral organs (Figs. 3.4 and 3.7). The three *A. x coerulea SEPALLATA* paralogs (*AqSEP1, AqSEP2,* and *AqSEP3*) are somewhat difficult to assess because they are variably expressed in control leaves but *AqSEP3* in particular seems to be up-regulated in *AqEMF2*-silenced leaves (Fig. 3.4B). These genes were not assessed in floral organs because they are already broadly expressed there. *A. x coerulea* also has three paralogs of the B class MADS box gene, *APETALA3* (*AqAP3-1, AqAP3-2,* and *AqAP3-3*). The petal-specific *AqAP3-3* locus is highly up-regulated in *AqEMF2*-silenced sepals, which also showed chimeric sepal/petal identity in several cases (Fig. 3.7A). Additionally two of the three *AP3* paralogs are moderately up-regulated in PRC2 VIGS treated leaves (Fig. 3.6B), but the expression of *AqAP3-1* and *AqAP3-2* is unaffected in mature sepals and petals (Fig. 3.7A and B). We also looked at the expression of *FUL-like 1* (*AqFL1*), which is normally expressed in early leaves, but no ectopic expression was detected (Fig. 3.7 and Fig. 3.6B).

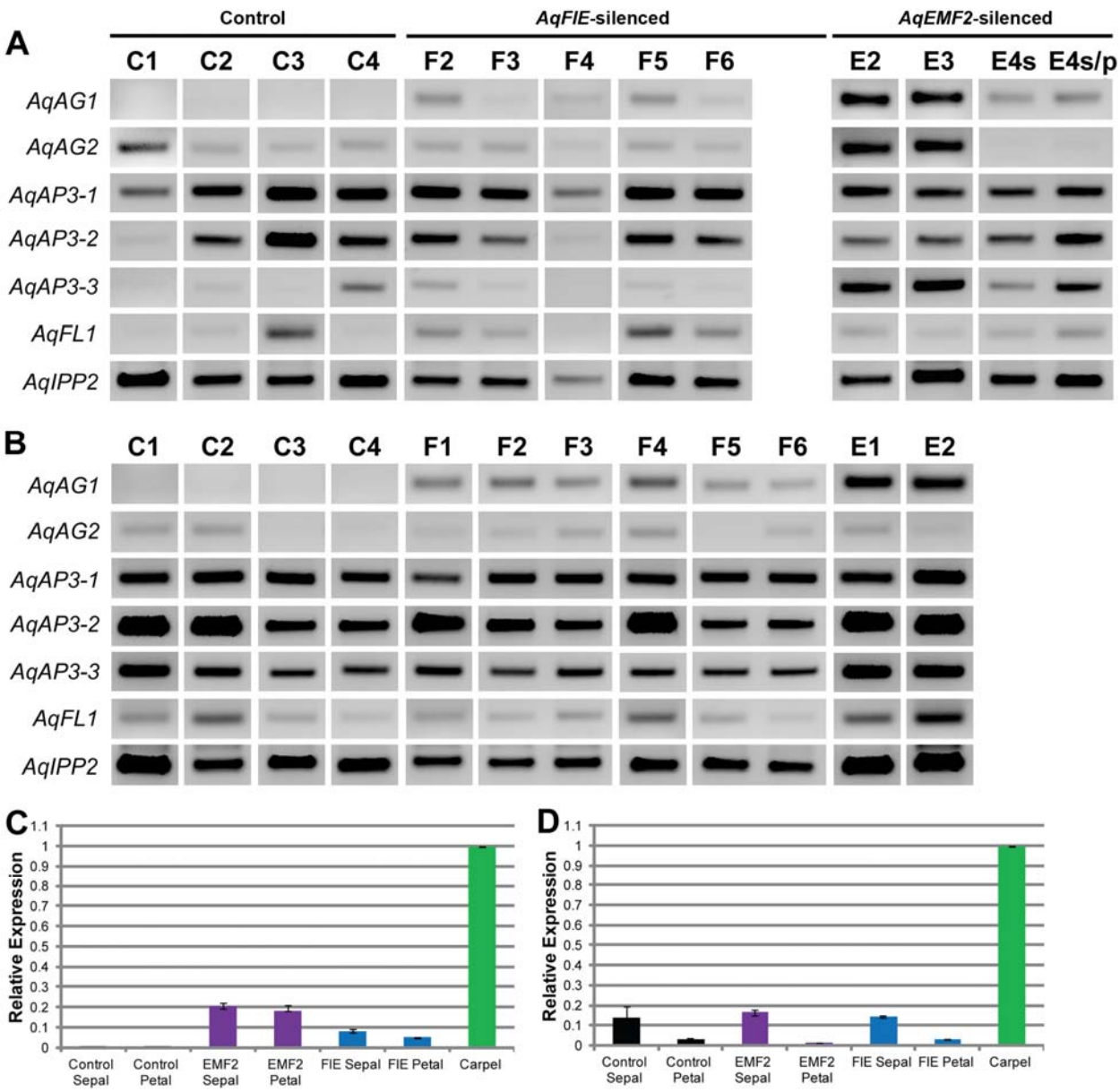


Figure 3.7: Expression of candidate genes in PRC2 VIGS treated floral organs. **A.** Expression of several floral organ identity genes in *AqANS*-silenced control sepals (C1-C4), and *AqFIE*- (F2-F6) and *AqEMF2*- (E1-E4s/p) treated first whorl organs. *AqAG1* is up-regulated in all *AqFIE*- and *AqEMF2*-treated organs compared to the controls.*AqAP3-3* also appears to be up-regulated in some of the sepals, particularly in *AqEMF2* down-regulated first whorl organs, several of which were in fact sepal/petal chimeras (s/p). Expression of *AqAP3-2* and *AqFL1* is variable in mature sepals and is difficult to assess. *AqAG2* and *AqAP3-1* expression does not

Figure 3.7 Continued: appear to be affected in silenced tissue. **B.** Expression of several floral organ identity genes in *AqANS*-silenced control petals (C1-C4), and *AqFIE*- (F2-F6) and *AqEMF2*- (E1-E4s/p) VIGS treated petals. *AqAG1* is up-regulated in all *AqFIE*- and *AqEMF2*-treated tissue compared to the controls. **C and D.** Quantitative Real Time PCR analysis of expression of *AqAG1* and *AqAG2* in *AqFIE, AqEMF2,* and *AqANS* control silenced tissue and wild type carpels. cDNA from two to four samples was pooled together prior to analysis. For each data point, three technical replicates were analyzed. *AqIPP2* expression was used for normalization. **C.** Average fold change in the expression of *AqAG1* in *AqFIE, AqEMF2,* and *AqANS* control silenced tissue normalized to wild type carpels with SD error bars. **D.** Average fold change in the expression of *AqAG2* in *AqFIE, AqEMF2,* and *AqANS* control silenced tissue normalized to wild type carpels with SD error bars.

Next, we tested for up-regulation of three of the five *A. x coerulea* class I KNOX genes. No significant ectopic KNOX gene expression could be detected in the leaves, although weak expression of *SHOOTMERISTEMLESS* 2 (*AqSTM2*) and *KNOTTED* (*AqKN*) is detected in *AqEMF2*-silenced leaves (Fig 3.4C). Given the lack of up-regulation in leaves and due to a limited amount of floral RNA, class I KNOX gene expression was not tested in the floral organs. Although *AqAG1* is consistently over-expressed in *AqFIE* and *AqEMF2* silenced sepals and petals, we never saw any evidence of carpel identity in these organs. We therefore pooled cDNA from several control, *AqFIE,* and *AqEMF2* petals and sepals and used qRT-PCR to further examined the expression of *AqAG1* and *AqAG2* in these organs as well as in wild type carpels (Fig. 3.7C and D). We found that while *AqAG1* was clearly up-regulated in *AqFIE* and *AqEMF2* silenced organs

compared to the controls, *AqAG1* expression was still much lower than in wild type carpels (about 0.05 to 0.2 fold). In contrast, *AqAG2* expression was similar in control and PRC2 silenced tissue, but much lower than in wild type carpels.

Lastly, in an effort to investigate the carotenoid production, we identified the likely *A. x coerulea* homologs of a range of components of the carotenoid pathway in *A. thaliana*, including enzymes involved in production (*PHYTOENE SYNTHASE* (*PSY*) and *CAROTENOID ISOMERASE* (*CRTISO*)) and breakdown (*CAROTENOID CLEAVAGE DIOXYGENASE 4* (*CCD4*) and *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*)) of carotenoids (Cazzonelli 2011). *A. x coerulea* has two copies *CCD4* (*AqCCD4* and *AqCCD4L)* and two genes that are closely related to *A. thaliana PSY* (*AqPSYL1* and *AqPSYL2*). Previous studies in *A. thaliana* have indicated that both *CRTISO* and *NCED3* are positively epigenetically regulated by other SET domain containing proteins so we were particularly interested in the expression of these genes in *AqFIE* and *AqEMF2* down-regulated tissue (Cazzonelli et al. 2010; Ding et al. 2011). We used RT-PCR to examine the expression of these six genes in *AqFIE* and *AqEMF2* silenced petals (Fig. 3.3B). Given the observed phenotypes, we might expect the expression of *AqPSYL1*, *AqPSYL2*, or *AqCRTISO* to be up-regulated or *AqCCD4, AqCCD4L*, or *AqNCED3* to be down-regulated. Unfortunately, no clear patterns are apparent from these reactions.

3.4: Discussion

AqFIE and *AqEMF2* VIGS treated plants displayed a range of lateral organ phenotypes. Silenced leaves often had ruffled or curled lamina, additional lobing, and an increased frequency of higher order branching. The perianth organs were generally narrower than wild type organs. Sepals were also curled and petals were stunted or had bent spurs while petal limbs also had a particularly intense yellow coloration seemingly due to an accumulation of carotenoid pigments in these cells. Many of the phenotypes we observed are similar to those seen in *clf* mutants and *FIE* cosuppressed *A. thaliana*, including curled leaves and narrow perianth organs (Goodrich et al. 1997; Katz et al. 2004). Unlike *clf* mutants and AG over-expressers in *A. thaliana,* dramatic transformation towards carpel identity was not observed in the *AqFIE* and *AqEMf2* down-regulated sepals or petals. However, the level of *AqAG1* expression in these organs was much less than what is seen in wild type carpels. Interestingly, the distinct folded morphology of the sepals may suggest slight transformation towards carpel identity as silenced leaves were folded towards the abaxial surface while the sepals were dramatically folded towards the adaxial surface, which is similar to the folding pattern of the *Aquilegia* carpel (Tucker and Hodges 2005).

It is interesting to note that in *AqFIE* silenced leaves, *AqEMF2* is also down-regulated. The reverse is not true in *AqEMF2* silenced leaves, and *AqEMF2* expression is not affected in *AqFIE* silenced floral organs. This result suggests that PRC2 may be directly or indirectly regulating *AqEMF2* expression in *A. x coerulea* leaves, which could account for the generally more severe phenotypes observed in *AqFIE* silenced leaves compared

to *AqEMF2* silenced leaves. *AqEMF2* is the only member of the complex that appears to be PRC2-regulated as the expression of *AqCLF* and *AqSWN* is not affected in PRC2 down-regulated leaves. In general, the potential for this type of cross-regulation is relatively unexplored in *A. thaliana* and, therefore, bears further study.

In our analysis of candidate target genes, we found that *AqAG1* is often ectopically expressed in PRC2 down-regulated tissue. *AqAP3-3* and *AqSEP3* are also up-regulated in some organs, but expression of the class I KNOX genes and several candidate genes involved in carotenoid production or degradation seem largely unaffected. Mutations in *AG* and *SEP3* are known to suppress the curled leaf phenotype in *clf* mutant plants while over-expression of these MADS box genes, which themselves function together in a complex (Honma and Goto 2001), is thought to be the cause of the curled leaf phenotype (Lopez-Vernaza et al. 2012). It is, therefore, possible that over-expression of *AqAG1* and *AqSEP3* is similarly responsible for many of the observed phenotypes in *AqFIE* and *AqEMF2* silenced leaves. These findings lead us to conclude that PRC2-based regulation of *AG* and *SEP3* homologs is deeply conserved in eudicots. It has recently been shown that several chromatin remodeling factors associate with MADS complexes and one model is that an important function of MADS domain complexes may be to recruit chromatin remodeling complexes to target loci in order to alter transcription of these genes and direct organ development (Immink et al. 2010; Smaczniak et al. 2012a). For example, RELATIVE OF EARLY FLOWERING 6 (REF6) was enriched in protein complexes that were isolated via immunoprecipitation using tagged ABCE class MADS box proteins (Smaczniak et al. 2012b). REF6 has been

shown to specifically demethylate H3K27me3, the histone modification deposited by PRC2 (Lu et al. 2011). Activation of *SEP3* by *APETALA1* (*AP1*) in *A. thaliana* results in the reduction of H3K27me3 at the *SEP3* promoter, suggesting that *AP1* may recruit *REF6* to the *SEP3* promoter in order to help induce *SEP3* gene function (Smaczniak et al. 2012b). Our data suggests that this key dependency on epigenetic regulation for the switch from vegetative to floral development may be important outside of *A. thaliana*. There are some complications, however. Of the two *A. x coerulea AG* homologs, only one, *AqAG1*, is strongly regulated by PRC2. These results suggest that PRC2 regulation can be directed in a paralog-specific fashion and may even play some role in the distinct expression patterns observed among these gene copies (Kramer et al. 2007).

The class I KNOX genes are directly or indirectly regulated by PRC2 in both *A. thaliana* and *Physcomitrella*, however, we did not detect any significant ectopic KNOX gene expression in our *AqFIE* and *AqEMF2* silenced leaves. This is somewhat surprising because of the increased frequency of higher order branching that we observed in silenced leaves. The class I KNOX genes are thought to play a role in compound leaf in a number of species. In compound leaf species where KNOX gene expression has been studied, it has been shown that they are expressed in the shoot apical meristem and down-regulated in incipient leaf primordia (P0), but subsequently turned back on in early leaf primordia (Bharathan et al. 2002). Down-regulation of KNOX genes in these leaves causes them to be less compound while over-expression of KNOX in compound leafed species leads to increased branching (Hareven et al. 1996; Hay and Tsiantis

2006), suggesting that KNOX genes act to maintain indeterminacy in compound leaves and promote leaflet initiation.

There are several explanations for why we did not observe ectopic KNOX gene expression in our VIGS treated leaves. First, it is possible the KNOX genes were ectopically expressed early in leaf development, but were later down-regulated by redundant mechanisms such as *ASYMMETRIC LEAVES 1* (*AS1*)-mediated repression (Phelps-Durr et al. 2005; Guo et al. 2008). However, it is important to remember that in other taxa with compound leaves, the KNOX and *AS1* homologs have lost their mutually exclusive regulatory interactions and are expressed together at later stages (Kim et al. 2003b). This may suggest that the *AS1*-dependent epigenetic silencing of KNOX genes that has been described in several simple-leafed models (Phelps-Durr et al. 2005; Guo et al. 2008) does not hold for plants with compound leaves. Along these lines, it is also possible that the increased branching phenotypes are due to other factors, such as accelerated phase change or novel genetic mechanisms regulating leaflet branching. For instance, a recent functional study of the gene *AqFL1* in *A. x coerulea* revealed that it promotes proper leaf margin development, a unique finding for homologs of this gene lineage (Pabón-Mora et al. 2013). This raises the possibility that factors other than the KNOX genes contribute to compound leaf branching in *Aquilegia*.

In addition to the conserved role in regulating *AG, AP3,* and *SEP3, A. x coerulea* PRC2 may target novel pathways, including those regulating carotenoid production or degradation. In *A. thaliana* patches of yellow anther-like tissue are observed on *clf*

mutant petals (Goodrich et al. 1997). However, the yellow pigmentation we observed is due to the accumulation of carotenoids in the plastids rather than to a partial homeotic transformation. While genes in the carotenoid pathway are not known to be suppressed by PRC2, some loci are positively epigenetically regulated in *A. thaliana*. Previous studies have shown that a major enzyme in the carotenoid biosynthesis pathway, CRTISO, requires the chromatin modifying enzyme SET DOMAIN GROUP 8 (SDG8) to maintain its expression (Cazzonelli et al. 2010). NCED3, an enzyme that cleaves some types of carotenoids as a part of abscisic acid (ABA) synthesis, is similarly epigenetically regulated by the *A. thaliana* trithorax homolog ATX1 (Ding et al. 2011). While none of the genes we tested were consistently up- or down-regulated in *AqFIE* and *AqEMF2* silenced petals, carotenoid production is very genetically complex and we were unable to test all of the candidate loci (Lu and Li 2008). Thus, it seems likely that PRC2 regulates an as yet unidentified enzyme in this pathway in *A. x coerulea.*

3.5: Conclusions

- A critical role for PRC2 in maintaining the repression of *AG*, *SEP3*, and possibly *AP3* appears to be conserved across eudicots. This conservation underscores the importance of chromatin remodeling factors in regulating the floral transition and the proper localization of floral organ identity.
- Class I KNOX genes are not ectopically expressed in PRC2 down-regulated tissue in *A. x coerulea*, possibly due to a regulatory shift associated with the evolution of compound leaves.

- *A. x coerulea* PRC2 plays a significant role in regulating the carotenoid pathway in floral organs, which has not been observed in other taxa.
- This study, the first to examine PRC2 function outside *A. thaliana* or the grasses, highlights how little we still know about the general conservation or targeting mechanisms underlying PRC2 function in major developmental transitions.

Chapter 4:

The *ASYMMETRIC LEAVES1* homolog in *Aquilegia x coerulea* controls KNOX gene expression and promotes laminar expansion in lateral organs.

4.1: Introduction

Coordination of cell division and expansion is essential for the proper development of multicellular organisms. In plants, a population of continually dividing, pluripotent stem cells located at the tips of growing shoots, known as the shoot apical meristem (SAM), provides the raw materials for plant development. Lateral organs such as leaves are formed from cells located along the flanks of the SAM. In order for these organs to develop properly, genes that promote pluripotency must be turned off in these cells while genetic networks that shape the organ are turned on. *ASYMMETRIC LEAVES 1* (*AS1*) is a R2-R3 class MYB transcription factor that is involved in both processes during leaf development (Stracke et al. 2001). *AS1* promotes cell determination and controls aspects of leaf shape in many angiosperms, including influencing leaf polarity and cell type differentiation, promoting proper laminar expansion and controlling leaflet placement in compound leaves (Waites and Hudson 1995; Schneeberger et al. 1998; Sun et al. 2002; Kim et al. 2003a; McHale and Koning 2004; Tattersall et al. 2005).

The first role for *AS1* in leaf development is the initial down-regulation of the class I KNOX genes in incipient primordia (Schneeberger et al. 1998; Byrne et al. 2000; McHale and Koning 2004). These homeodomain-containing transcription factors are expressed in the SAM but must be turned off in developing leaf primordia to allow for proper leaf development (Jackson et al. 1994; Chuck et al. 1996; Bharathan et al. 2002). In simple-leafed taxa, *AS1* homologs are expressed in a complementary pattern to the class I KNOX: no *AS1* expression is seen in the SAM where KNOX genes are strongly expressed, but *AS1* expression is detected very early in leaf initiation and

throughout leaf development while the KNOX genes are silenced (Waites et al. 1998; Tsiantis et al. 1999; Byrne et al. 2000; McHale and Koning 2004). Consistent with this, in *Arabidopsis thaliana as1* mutants, leaf phenotypes resemble KNOX gene over-expressing lines including downwardly curling leaves, leaves with extra lobes, aberrant vascular patterning, and ectopic shoots on the adaxial surface of the petiole (Chuck et al. 1996; Byrne et al. 2000). Three *A. thaliana* class I KNOX genes, *BREVIPEDICELLUS* (*BP*) and *KN-LIKE IN ARABIDOPSIS THALIANA 2* and *6* (*KNAT2* and *KNAT6)* are ectopically expressed in *as1* leaves (Byrne et al. 2000; Semiarti et al. 2001; Hay and Tsiantis 2009). Mutations in *AS1* homologs in other species also cause ectopic KNOX gene expression, as well as similar leaf phenotypes, including *nsphan* in *Nicotiana* (tobacco) and *rough sheath2* (*rs2*) in *Zea* (maize) (Tsiantis et al. 1999; McHale and Koning 2004).

When targeting the KNOX loci, AS1 forms a complex with several other proteins. The most important of these appears to be the LOB domain containing protein, ASYMMETRIC LEAVES 2 (AS2), which interacts with AS1 and has many of the same mutant phenotypes as *as1*, including ectopic expression of *BP, KNAT2* and *KNAT6* and loss of KNOX gene expression in *AS2* over-expression lines (Semiarti et al. 2001; Lin et al. 2003; Xu et al. 2003; Phelps-Durr et al. 2005). The AS1-AS2 complex binds directly to several KNOX gene promoters in *A. thaliana* and both AS1 and AS2 are required for these interactions (Guo et al. 2008). In maize and *A. thaliana*, AS1 also interacts with HIRA, a chromatin remodeling factor that promotes gene silencing (Phelps-Durr et al. 2005). Both *BP* and *KNAT2* are ectopically expressed in *HIRA* co-suppression lines and

it has been suggested that the AS1-AS2 complex silences KNOX genes by recruiting HIRA to the KNOX promoter and forming a repressive chromatin state (Phelps-Durr et al. 2005; Guo et al. 2008). The AS1-AS2-HIRA complex likely interacts with other proteins to mediate KNOX gene silencing including several TCP transcription factors and JAGGED LATERAL ORGANS (JLO) (Borghi et al. 2007; Li et al. 2012).

In addition to its role in leaf initiation, the AS1-AS2 dimer also regulates aspects of leaf polarity and laminar outgrowth in a number of species. Leaves are initiated as radially symmetric bulges on the sides of the meristem, but later acquire asymmetry along three major axes; medial-lateral, proximal-distal, and abaxial-adaxial (dorsal-ventral) (Reviewed in: Moon and Hake 2011). While maize and *A. thaliana rs2* and *as1* mutants are reported to have defects in proximal-distal patterning, (Schneeberger et al. 1998; Tsiantis et al. 1999; Sun et al. 2002), the genes are also implicated in abaxial-adaxial polarity, which is important in leaf development. The adaxial surface is closest to the SAM and is specialized for photosynthesis, while the abaxial surface has a high stomatal density and is specialized for gas exchange (Reviewed in: Moon and Hake 2011; Yamaguchi et al. 2012). Juxtaposition between abaxial and adaxial identity in the leaf is thought to be essential for laminar outgrowth (Waites and Hudson 1995) (Reviewed in: Yamaguchi et al. 2012) and AS1/AS2 function together in one of several pathways that establishes adaxial identity in the developing leaf. The degree to which this role is redundant with other pathways varies greatly across the angiosperms. In *Antirrhinum,* for example, the mutant phenotype of the *AS1* homolog *phantastica (phan)* is largely due to alterations in abaxial-adaxial polarity (Waites and Hudson 1995; Waites

et al. 1998). Weak *phan* mutant leaves are narrow or heart shaped and have patches of abaxial tissue on the adaxial surface that are surrounded by ridges of ectopic laminar outgrowth where the two identities meet, while strong mutants have needle-like leaves that are completely abaxialized (Waites and Hudson 1995). Although defects in abaxial-adaxial polarity were not initially observed maize and *A. thaliana rs2* and *as1* mutants (Schneeberger et al. 1998; Byrne et al. 2000), *A. thaliana AS2* is expressed only on the adaxial surface of leaf primordia (Iwakawa et al. 2002), which thereby limits AS1/AS2 function to the upper side of the leaf. It was later discovered that *AS2* over-expression lines (Iwakawa et al. 2002; Lin et al. 2003; Xu et al. 2003), as well as mutants in the maize *AS2* homolog *INDETERMINATE GAMETOPHYTE 1* (*IG1*) (Evans 2007), show defects in abaxial-adaxial polarity, suggesting that this polarity role for the AS1/AS2 dimer may be conserved in both monocots and dicots.

The variable importance of *AS1* homologs to establishing abaxial-adaxial polarity is in part due to genetic interactions between the AS1/AS2 complex and several other factors involved in leaf polarity. These include the additional abaxial factors *REVOLUTA (REV), PHABULOSA (PHB),* and *PHAVOLUTA (PHV)* and the complementary abaxial identity genes, *KANADI1 (KAN1), KANADI2 (KAN2), FILAMENTOUS FLOWER (FIL),* and *YABBY3 (YAB3)*, all of which show perturbed expression in mutant or over-expression *AS1/AS2* backgrounds (Lin et al. 2003; Fu et al. 2007b). Studies are still seeking to understand how the juxtaposition of abaxial and adaxial identity promotes laminar outgrowth, but recently members of the WOX family of homeodomain transcription factors were implicated in maintaining the central meristematic domain that

drives this process (Nardmann et al. 2004; Vandenbussche et al. 2009; Tadege et al. 2011a; Nakata et al. 2012). Furthermore, these WOX genes may also play a role in maintaining abaxial-adaxial identity in leaf margin where they appear to interact genetically with the *AS1* and *AS2* (Vandenbussche et al. 2009; Nakata et al. 2012). In *A. thaliana*, *AS2* was shown to be ectopically expressed on the abaxial surface of older leaflets in *PRESSED FLOWER /WOX1* double mutants suggesting that the WOX genes are acting to restrict *AS2* expression during laminar expansion (Nakata et al. 2012). However, changes in *AS1* and *AS2* expression have not been observed in other species and it is unclear if the AS1-AS2 dimer regulates WOX expression during the establishment of the abaxial-adaxial boundary (Vandenbussche et al. 2009; Tadege et al. 2011a).

The functions of *AS1* are understood simple leaf development, but *AS1* has been less well studied in species with compound leaves. Simple leaves have a single flat lamina while compound leaves are composed of multiple regularly spaced blades, known as leaflets, arranged along or around a central rachis, which can take a number of different patterns (Reviewed in: Efroni et al. 2010). In pinnate leaves, the leaflets are positioned along the sides of the rachis while in palmate leaves the leaflets are clustered at the tip of the rachis, which may also be simply referred to as the petiole (Kim et al. 2003a). Palmate leaves are further categorized into peltately palmate leaves, in which the leaflets are attached around the entire circumference of the petiole terminus and non-peltately palmate leaves, in which no leaflet is present on the adaxial side of the petiole terminus (Kim et al. 2003a). In several compound-leafed models, *AS1* homologs appear

to be involved in both initiating leaflets within the leaf primordia and in determining leaflet arrangement along or around the rachis. This role is likely related to the broadly conserved regulatory interactions between *AS1* and the class I KNOX genes. Unlike class I KNOX genes in simple-leafed species the class I KNOX genes of compound leafed taxa are reactivated in developing leaves, after being initially down-regulated at primordium initiation (Bharathan et al. 2002). Over expression of several class I KNOX in tomato or the distantly related *Cardamine*, a close relative of *A. thaliana*, results in increased leaflet number and branching within the leaf (Hareven et al. 1996; Hay and Tsiantis 2006; Jasinski et al. 2007). Taken together this suggests that KNOX genes may act to maintain indeterminacy in compound leaves and promote leaflet initiation, even in lineages that have independently evolved compound leaves. Consistent with these findings, in these taxa the *AS1* homologs are often co-expressed with the class I KNOX genes in developing leaves, suggesting a new type of regulatory interaction (Koltai and Bird 2000; Kim et al. 2003a). Functional studies in tomato indicate that cooperative functions of the *AS1* and KNOX homologs regulate positioning of leaflet initiation and laminar expansion such that complete loss of *AS1* function results in simple leaves with major polarity defects (Kim et al. 2003a; Kim et al. 2003b). In addition, moderate loss of AS1 function perturbs the positioning of leaflets, resulting in loss of rachis polarity and the development peltately palmate compound leaves (Kim et al. 2003a). Many of these roles for *AS1* homologs are observed in studies of other compound-leafed models, such as *Cardamine* and pea (Tattersall et al. 2005; Hay and Tsiantis 2006).

Here we attempt to further our understanding of *AS1* function in compound leaf development by investigating *AS1* expression and function in *Aquilegia.* As a member of the order Ranunculales, an early diverging lineage of the eudicot angiosperms that arose before the radiation of the core eudicots, the genus *Aquilegia* represents an independent evolution of compound leaves as well as a rough phylogenetic midpoint between *A. thaliana* and model systems in the grasses (Reviewed in: Kramer and Hodges 2010). Furthermore, unlike tomato, *Cardamine,* and pea, *Aquilegia* leaves are palmate rather than pinnate, although some conflict exists as to whether they are peltately or non-peltately palmate (see below). Historically, *Aquilegia* has been used for ecological, evolutionary and genetic studies. It has a number of useful features including a small, sequenced genome (n=7, approximately 300 Mbp) (http://www.phytozome .net/search.php?method=Org_Acoerulea) (Reviewed in: Hodges and Kramer 2007; Kramer 2009). Additionally virus-induced gene silencing (VIGS), a reverse genetics tool that utilizes the RNAi pathway has been optimized in several species of *Aquilegia* and used to study both leaf and floral development (Gould and Kramer 2007; Kramer et al. 2007; Sharma et al. 2011; Pabón-Mora et al. 2013; Sharma and Kramer 2013). Recent studies of leaf development in *Aquilegia* have revealed patterns of both genetic conservation and novelty. It appears that a genetic program depending on the *CUP-SHAPED COTYLEDON* (*CUC*) genes does play a conserved role in promoting marginal lobing in *Aquilegia* (Blein et al. 2008), but the MADS box gene *AqFL1,* whose homologs typically promote floral meristem identity, has been found to also contribute to lobing of the leaf margin (Pabón-Mora et al. 2013).

In this study, we have sought to characterize the expression patterns of *AS1* and *AS2* homologs in *Aquilegia* and to characterize the knock-down phenotype of *AqAS1*. These analyses, together with studies of candidate interacting loci, has provided much greater insight into both the conservation of *AS1* lineage function and the novel aspects of leaf development that may be at work in *Aquilegia*.

4.2: Methods

Gene Cloning

In order to identify genes of interest in the *Aquilegia* genome, BLAST searches (Altschul et al. 1990) of the *Aquilegia* DFCI Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=*Aquilegia*) and the *Aquilegia coerulea* genome (http://www.phytozome.net/search.php?method=Org_Acoerulea) were performed using *A. thaliana* sequences. In the case of *AqAS2,* the annotated transcript appeared to be incomplete, so 3' Rapid Amplification of cDNA Ends (3' RACE) was used to determine the complete cDNA sequence. 3' RACE was performed using a mix of cDNA prepared from RNA isolated from young leaves (see Appendix 1 for primer sequences) as described in Kramer et al. (2003). Fragments were cloned using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) and Z-Competent *E. coli* cells strain c-600 (Zymo Research, Irvine,CA) and several clones were sequenced. Primer extension sequencing was performed by GENEWIZ, Inc. (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. The reactions were then run on Applied Biosystem's 3730xl DNA Analyzer.

Phylogenetic Analysis

For all gene trees, homologs of *AS1* and *AS2* were identified from a variety of land plant taxa by using the BLAST algorithm to search GenBank, the DFCI Plant Gene Index (http://compbio.dfci.harvard.edu/tgi/plant.html) and Phytozome (http://www.phytozome.net/), or through literature searches. For all datasets, amino acid sequences were initially aligned using Clustal W and then adjusted by hand using MacVector (Cary, North Carolina). Maximum likelihood analysis was completed using RAxML (Stamatakis et al. 2008) as implemented by the CIPRES Science Gateway (http://www.phylo.org/portal2/login!input.action) (Miller et al. 2010). The model of amino acid evolution used was the default JTT. Bootstrap values are presented at all nodes with greater than 50% support.

In situ Hybridization

Suitable probe template fragments of each gene were amplified from *Aquilegia vulgaris* or *Aquilegia coerulea* cDNA using PCR (see Appendix 1 for primer sequences). Different species were used simply due to varying availability at the time the probes were prepared. These regions were designed so that they did not include highly conserved domains. Note that the extremely high sequence conservation among *Aquilegia* species (Whittall and Hodges 2007) allows the use of probe templates derived from different species without difficulty (Zhang et al. 2013). The PCR products were cloned using the TOPO-TA Cloning Kit and TOP10 *E.coli* competent cells (Invitrogen, Carlsbad, CA). Inflorescences and vegetative meristems at the 1-2 true leaf stage were collected from *Aquilegia coerulea.* Tissue was fixed under vacuum in FAA, dehydrated,

and embedded in Paraplast (Lecia Biosystems, Wetzlar, Germany). *In situ* hybridization was preformed as described in Kramer (2005). See Table 2 for hydrolysis lengths. Results were visualized in the Harvard Center for Biological Imaging on a Zeiss AxioImager Z2 microscope using trans-illumination with white light. Images were taken using a Zeiss AxioCam Mrc digital camera.

Table 2 – Hydrolysis lengths of *in situ* probes.

Gene	Full Length	Hydrolyzed Length
AqAS1	146 bp	x
AqAS2	310 bp	155 bp
AqHIS4	331 bp	150 bp
AqKN	473 bp	75 bp
AqSTM1	308 bp	200 bp
AqSTM2	327 bp	x

Virus-Induced Gene Silencing

The *Aquilegia* VIGS protocol was preformed as described previously (Gould and Kramer 2007). The TRV2-*AqAS1-AqANS* construct was made by PCR amplifying an approximately 300 bp region of the gene using primers that added *Eco*R1 and XbaI restriction sites to the 5' and 3' ends of the PCR product (see Appendix 1 for primer sequences). The PCR products were then purified and cloned into the pre-existing TRV2-*AqANS* construct (Gould and Kramer 2007) and electroporated into *Agrobacterium* cultures. *Aquilegia* seedlings were grown to approximately the 4 to 6 leaf stage and then either treated as described in Gould and Kramer, 2007 to yield

unvernalized samples or vernalized for approximately 4 weeks at 4^{o}C and then treated as described in Sharma and Kramer (2013). Leaves, petals, and sepals showing *AqANS* silencing were photographed, collected, and stored at -80^{o}C for RNA analysis.

qRT PCR

To asses expression of *AqAS1* and *AqAS2* throughout the life cycle of *Aquilegia*, the following tissue was collected from *Aquilegia coerulea* plants: whole seedlings at the cotyledon stage, young leaves from 4-6 leaf stage unvernalized plants, meristems from 4-6 leaf stage unvernalized plants (pre-vern meristems), meristems subjected to 4 weeks of cold treatment at 4°C (post-vern meristems), inflorescence meristems and anthesis stage sepals, petals, stamens, staminodia, and carpels. At each stage, samples from three different plants were collected and pooled. Total RNA was extracted using either the RNeasy Mini kit (Qiagen, Valencia, CA) (leaves, pre- and post-vern meristems, and inflorescences) or the Pure-Link Plant RNA Reagent small scale RNA isolation protocol (Ambion, Austin, TX) (whole seedlings and anthesis stage floral organs). RNA was treated with Turbo DNase (Ambion, Austin, TX) and cDNA was synthesized from 1 µg of total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT) primers. cDNA was diluted 1:50 prior to use.

To assess gene expression in *AqAS1* VIGS treated tissue, RNA was extracted from wild type, control (*AqANS* silenced), and experimental (*AqAS1* VIGS treated) tissue. RNA was extracted using the Pure-Link Plant RNA Reagent small scale RNA isolation protocol (Ambion, Austin, TX), DNased, and used to synthesize cDNA as described

above. cDNA was diluted 1:5 then pooled and diluted 1:10. Petal and sepal pools contained cDNA from 4-6 individuals. Leaf pools contained cDNA from 8 individuals. qRT-PCR was performed using PerfeCTa qPCR FastMix, Low ROX (Quant Biosciences Inc., Gaithersburg, MD) in the Stratagene Mx3005P QPCR system to study the relative expression of *AqAG1* and *AqAG2. AqIPP2* expression was used for value normalization. All primers are listed in Appendix 1.

Histology

All histology was preformed as described in Ruzin (1999). Leaves and petioles were collected from control (*AqANS* silenced, also referred to as WT) and experimental (*AqANS-AqAS1* VIGS treated, simply referred to as *AqAS1* treated) tissue, fixed under vacuum in FAA, dehydrated, and embedded in Paraplast (Lecia Biosystems, Wetzlar, Germany) (see: Kramer 2005). Samples were sectioned to 8 mm with a disposable steel blade on a Reichert–Jung microtome (Lecia Biosystems, Wetzlar, Germany). Staining was preformed as described in Ruzin (1999). Two staining protocols were used. To visualize tissue and cell morphology, sections were deparaffinized, rehydrated, and stained in a 1% aqueous Safranin O solution for approximately 90 minutes, then dehydrated and stained with 5% Fast Green and 95% ethanol for approximately 30 seconds. To visualize lignified tissues, particularly vascular bundles, sections were deparaffinized, washed in 100% ethanol and then stained in 2% phloroglucinol, which specifically stains lignin. Results were visualized in the Harvard Center for Biological Imaging on a Zeiss AxioImager Z2 microscope using trans-illumination with white light. Images were taken using a Zeiss AxioCam Mrc digital camera.

For dark field microscopy, whole leaves were collected from control (*AqANS* silenced) and experimental (*AqAS1* VIGS treated) tissue and fixed under vacuum in FAA, dehydrated, and cleared in CitriSolv (Fisher Scientific, Waltham, MA) over night. Tissue was imaged in the Harvard Center for Biological Imaging using a Kontron Elektronik ProgRes 3012 digital camera mounted on a Leica WILD M10 dissecting microscope.

Scanning Electron Microscopy

AqAS1 VIGS and control *ANS* VIGS petal and leaf samples were fixed in FAA and stored at 4^{o}C for a period of at least 48 hours. They were then partially dehydrated in a graded ethanol series to 70% EtOH and stored at 4^{o}C. Two days prior to critical point drying, the graded ethanol series was continued to transition the samples to 100% EtOH. Samples were dried with a Tousimis Auto Samdri 815 Series A critical point dryer and sputter-coated with gold-palladium using a Cressington HR 208 sputter coater with an accelerating current of 20uA. Imaging was carried out on a Zeiss EVO 55 Environmental SEM at an accelerating current varying from 20 to 21 kV.

4.3: Results

Homologs of *AS1* and *AS2* in the *Aquilegia* Genome

We identified putative *AS1* and *AS2* homologs in the *Aquilegia coerulea* genome (http://www.phytozome.net/) using BLAST searches and confirmed their identity via Maximum Likelihood (ML) analysis (Fig. 4.1). *AqAS1* was placed with a high degree of confidence within a large clade containing the identified *AS1* orthologs *ZmRS2*,

LePHAN, *AmPHAN*, and *AtAS1* (Fig. 4.1A). We also analyzed a subset of the class I LOB domain family and identified one *Aquilegia* gene, termed *AqAS2* that fell within a well-supported clade that contained several known *AS2* homologs (Fig. 4.1B).

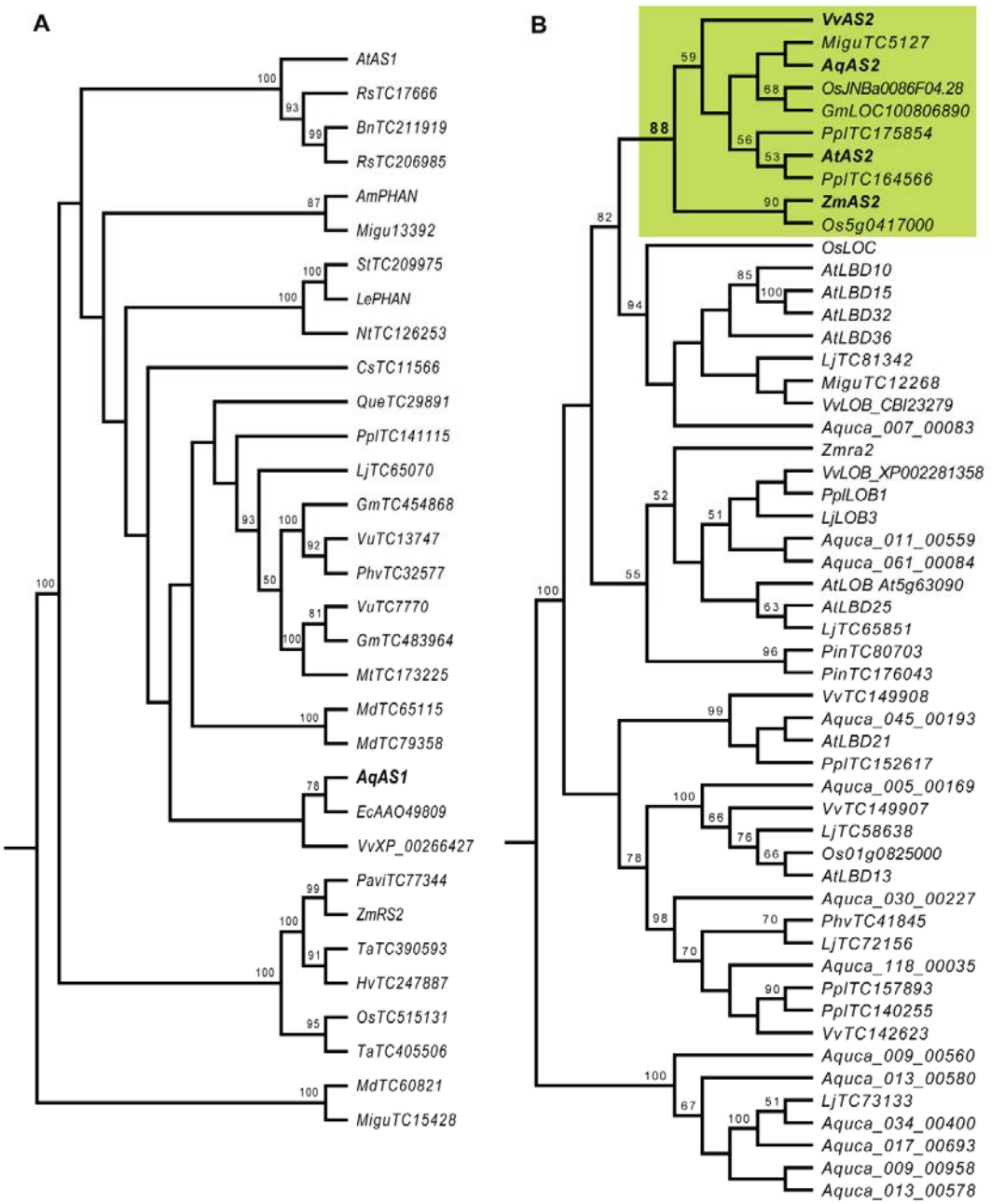


Figure 4.1: Identification of *Aquilegia AS1* and *AS2* homologs. **A.** Maximum likelihood (ML) analysis of *ASYMMETRIC LEAVES 1* (*AS1*) homologs with ML bootstrap values shown at the nodes. Boostrap values less than 50 have been omitted. **B.** Maximum likelihood (ML) analysis of several clades of the class I Lateral Organ Boundaries (LOB) domain family with ML bootstrap values shown at the nodes. Bootstrap values less than 50 have been omitted. *ASYMMETRIC LEAVES 2* (*AS2*) clade highlighted in green.

Expression Analysis of *AqAS1* and *AqAS2* in *A. coerulea*

Tissue was collected at different stages throughout the life cycle of *Aquilegia coerulea* and qRT-PCR was used to assess the expression of *AqAS1* and *AqAS2* (Fig. 4.2). Three technical replicates were analyzed for each primer set on each sample and the data was normalized relative to the expression of the housekeeping gene *AqIPP2*. *AqAS1* expression is highest in leaves, unvernalized and vernalized meristems and in the inflorescence and lowest in anthesis stage floral organs. *AqAS2* expression peaks in vernalized meristems and is also high in the inflorescence and in anthesis stage sepals.

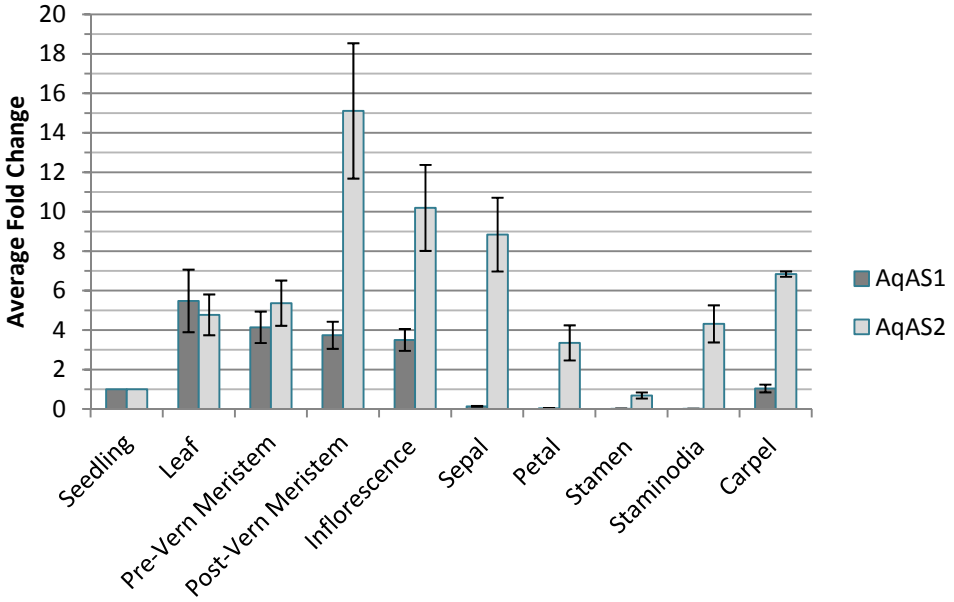


Figure 4.2: qRT-PCR analysis of *AqAS1* and *AqAS2* expression in *A. coerulea.* The graph shows average fold change for each stage normalized to whole seedlings with SD error bars. Tissue from three plants was collected and pooled at each stage. For each data point, three technical replicates were analyzed. *AqIPP2* expression was used for normalization.

In Situ Hybridization of *AqAS1, AqAS2*, and the class I KNOX genes in *Aquilegia*

In order to further characterize the expression of *AqAS1* and *AqAS2*, we performed *in situ* hybridization on vegetative and inflorescence meristems (Fig. 4.3). *AqAS1* and *AqAS2* have similar expression patterns in that both genes are expressed in the vegetative meristem and in the tips of developing leaflets, but expression appears to be highest the tips of young leaves (Fig. 4.3A-F). While the expression of *AqAS1* appears concentrated in the medial zone of older leaflets (Fig. 4.3C), neither gene was clearly asymmetrically expressed along the abaxial/adaxial axis (data not shown). We also saw moderate expression of *AqAS1* and *AqAS2* in the distal portions of developing floral organs (Fig. 4.3G-H).

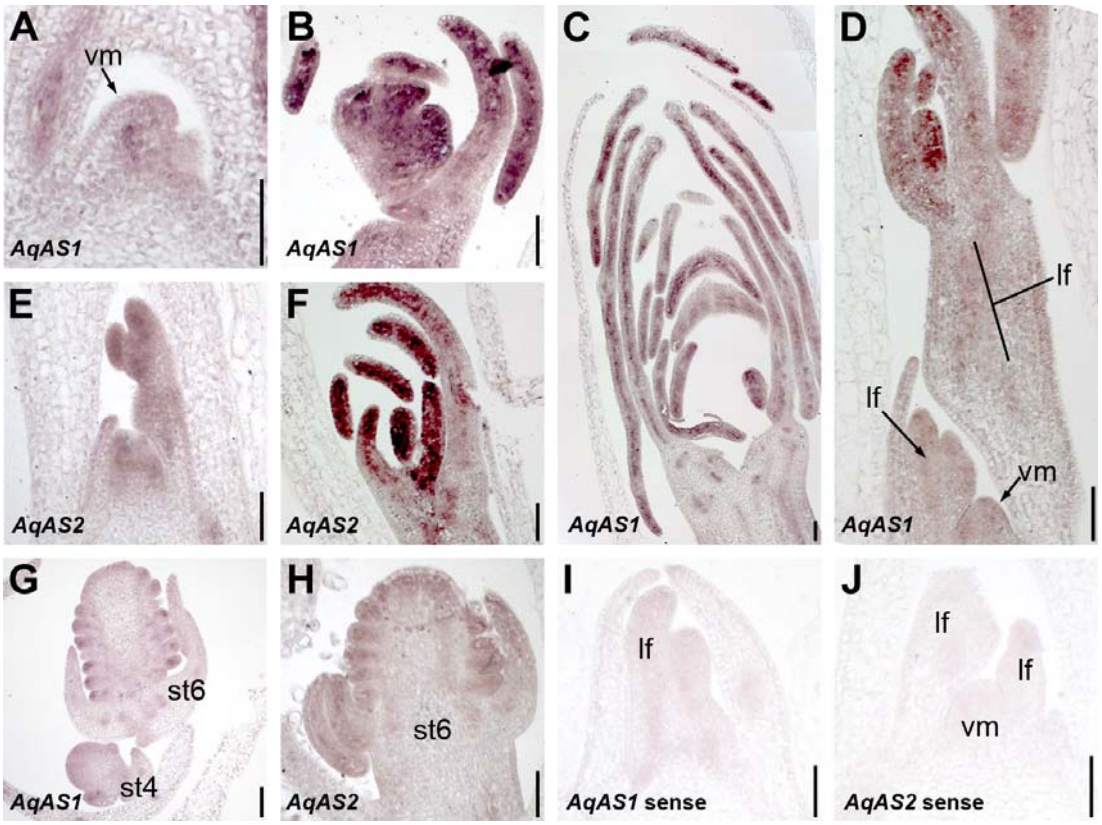


Figure 4.3: *In situ* hybridization of *AqAS1* and *AqAS2* in vegetative and floral meristems. **A-D.** *AqAS1* expression in vegetative tissue. **A.** *AqAS1* is detected throughout the vegetative meristem (vm). **B.** *AqAS1* expression in young leaflets. Expression is higher near the tips of each leaflet. **C.** *AqAS1* expression in older leaflets. Expression appears concentrated in the medial zone of the leaflets. **D.** *AqAS1* expression in a vegetative meristem (vm) and young leaf (lf). Expression is higher in the leaf than in the meristem. **E-F.** *AqAS2* expression in vegetative tissue. **E.** *AqAS2* expression is higher in the young leaves than in the vegetative meristem. **F.** *AqAS2* expression in older leaves also appears to be highest in the medial zone of each leaflet. **G.** *AqAS1* expression in stage 4 and stage 6 floral meristems. Expression is seen throughout the younger meristem. In the older meristem *AqAS1* expression is concentrated in the tips of the initiating floral organs. **H.** *AqAS2* is expressed in the initiating floral organs of a stage 6 floral meristem. **I.** *AqAS1* sense probe in young a young leaf. **J.** *AqAS2* sense probe in a vegetative meristem. Scale Bars: 100µm.

In order to begin to understand the regulatory interactions between AqAS1/AS2 and the major class I KNOX genes, we also examined the expression of three of the *Aquilegia* class I KNOX genes, *AqSTM1, AqSTM2,* and *AqKN*, in vegetative meristems (Fig. 4.4). *AqSTM1* is expressed throughout the meristem and at the very tips of newly initiated leaves (Fig. 4.4A-B). In some cases, *AqSTM1* expression appears to be down-regulated in a sector of the meristem, most likely in association with the initiation of new leaf primordia (P0) (Fig. 4.4B). *AqSTM1* expression is also seen at the tips of older leaves as the leaflets begin to initiate and in the expanding leaflets (Fig. 4.4C-D). *AqSTM2* and *AqKN* are also expressed in the meristem and in developing leaves, although at levels that seem closer to background and are, therefore, hard to visualize clearly (Fig. 4.4E-I). *AqKN* is particularly expressed at higher levels in the tips of recently initiated leaflets (Fig. 4.4H). Overall, the highest levels of class I KNOX expression overlap with tissues that continue to proliferate in the developing leaf, as visualized by *AqHIS4* expression (Fig. 4.4J-K).

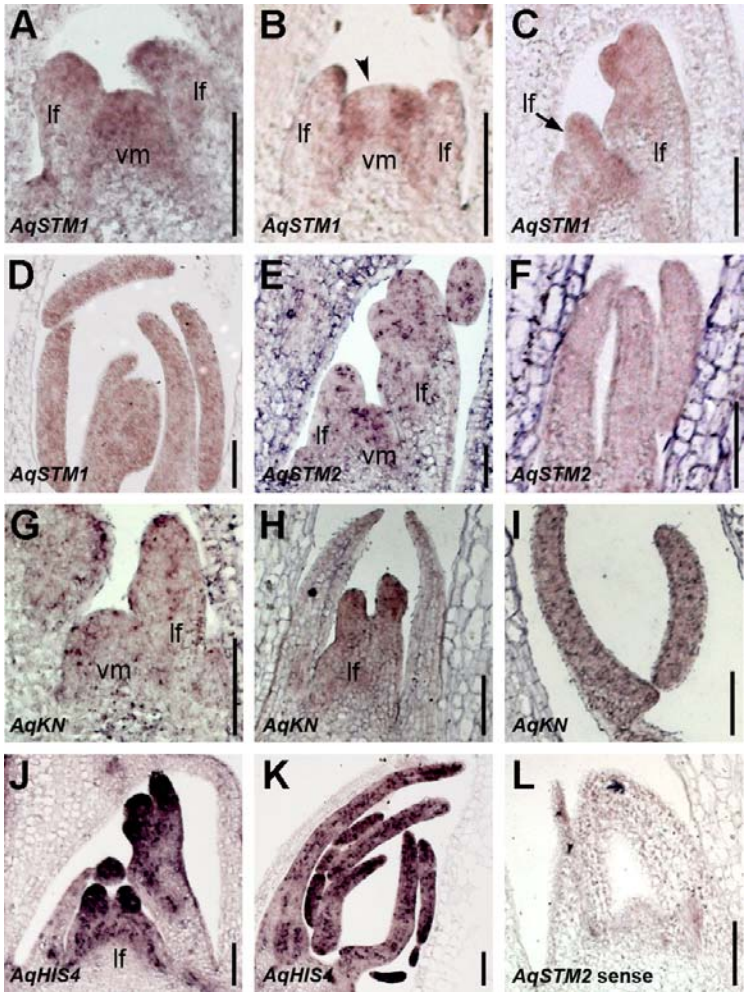


Figure 4.4: *In situ* hybridization of class I KNOX genes and *AqHIS4* in vegetative meristems. **A-D.** *AqSTM1* expression in vegetative tissue. **A.** *AqSTM1* is expressed throughout the vegetative meristem (vm) and at the tips of young leaves (lf). **B.** *AqSTM1* expression is lost in a portion of the meristem (arrowhead), which may correspond to an incipient leaf primordia. **C.** *AqSTM1* expression is also seen in the tips of the leaves after leaflet formation has begun. **D.** *AqSTM1* expression in older leaflets. **E-F.** *AqSTM2* expression in vegetative tissue. **E.** *AqSTM2* is expressed at low levels throughout the meristem (vm) and in the tips of young leaves (lf). **F.** *AqSTM2* expression in older leaflets. **G-I.** *AqKN* expression in vegetative tissue. **G.** Weak *AqKN* expression is seen throughout the vegetative meristem (vm) and young leaves (lf). **H.** *AqKN* expression appears highest in the tips of initiating leaflets. **I.** *AqKN* expression in older leaflets.

Figure 4.4 Continued: J-K. *AqHIS4* expression in vegetative tissue. **J.** *AqHIS4* expression is highest in the tips initiating leaflets, coincident with the highest KNOX gene expression. **K.** *AqHIS4* expression in older leaflets. **L.** *AqSTM2* sense probe in vegetative tissue. Scale Bars: 100μm.

Characterization of *AqAS1* Silenced Plants – Vegetative Phenotypes

We treated approximately one hundred unvernalized and more than one hundred and fifty vernalized plants with a TRV2 construct containing *AqAS1* fragments and treated an equivalent number with a TRV2 construct containing *AqANS* alone as a control. As is common for VIGS-treated plants, we recovered a range of phenotypes (Fig. 4.5) (Gould and Kramer 2007). Wild type *Aquilegia* leaves are compound, typically bearing three leaflets that are themselves divided into two to three lobes (Fig. 4.5A). However, *Aquilegia* does display heteroblasty over the course of its lifespan, with varying leaf morphology as the individual progresses from the vegetative to the reproductive stage (See Appendix 3). Using the terminology of Kim et al. (2003a), all of these leaf forms are non-peltately palmate in that the leaflets are not radially positioned around the terminus of the primary petiole, although the petiole itself is quite radial.

Leaves of *AqAS1* treated plants were curled towards the abaxial side of the leaf with the most severely affected leaves being almost completely cylindrical (Fig. 4.5B-H). The veins on these curled leaves appeared more prominent than controls, protruding markedly above the plane of the lamina (Fig. 4.5B-C, E-F). The leaves were also more deeply lobed than wild type (Fig. 4.5B-F). In one case, the medial lobes of each leaflet

were completely radialized (Fig. 4.5I). Occasionally the leaflets were arranged in a whorl around the terminus of the petiole (peltately palmate) instead of the usual arrangement (non-peltately palmate) (Fig. 4.5G and J) (Kim et al. 2003a).

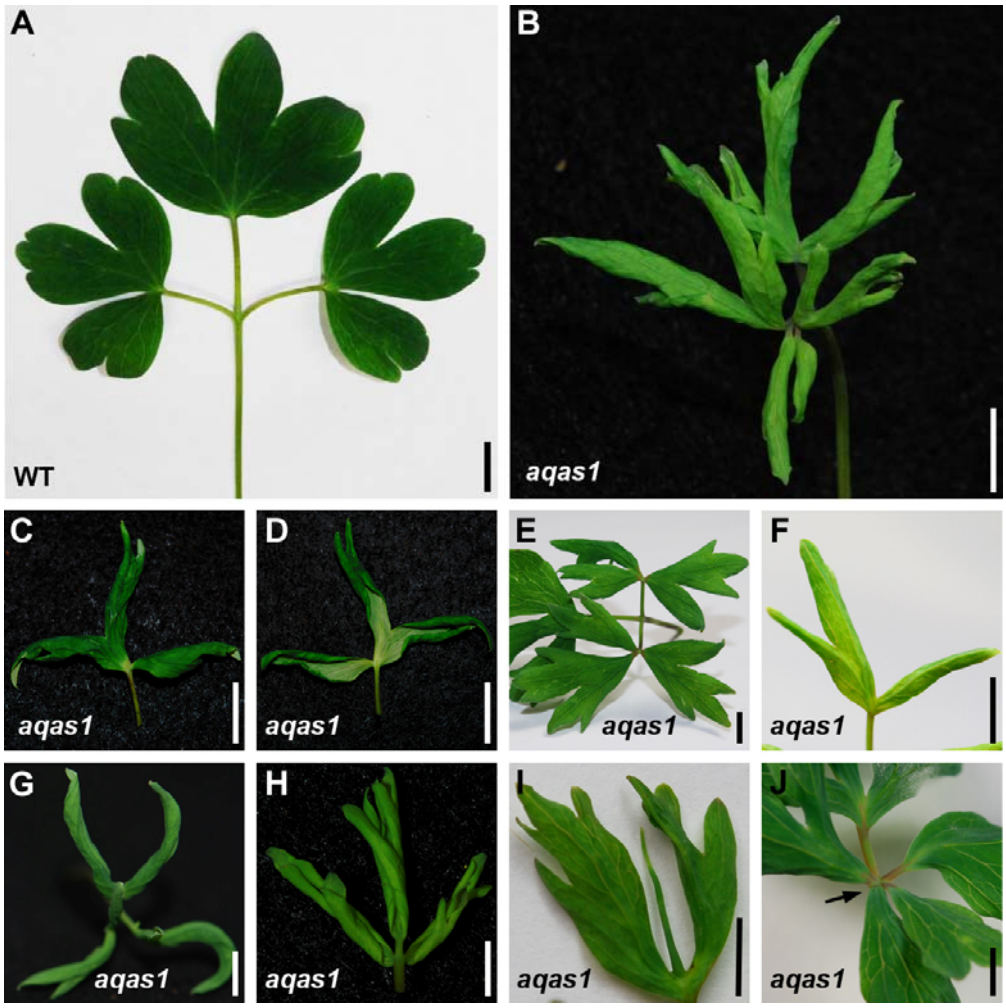


Figure 4.5: *AqAS1* VIGS vegetative phenotypes. **A.** Wildtype leaf with three lobed leaflets. **B-J** *AqAS1* VIGS treated leaves and leaflets. **B.** Entire leaf with highly curled leaves. **C.** Adaxial side of a leaflet showing highly curled lamina and deep lobes. **D.** Abaxial side of the same leaflet. **E.** Weakly silenced leaf with deep lobes and prominent vasculature. **F.** Deeply lobed leaflet. **G.** Leaf with extremely curled leaflets that are arranged in a whorl around the petiole terminus.

Figure 4.5 Continued: H. Abaxial side of a moderately curled leaflet. **I.** Leaflet with a completely radialized central lobe. **J.** Leaflets arranged in a whorled pattern around the petiole terminus. Scale Bars: 1cm.

We further characterized these vegetative phenotypes using various histological techniques and Scanning Electron Microscopy (SEM) (Fig. 4.6). Cross sections of *AqAS1* treated leaves showed that the cells of the adaxial surface were smaller and more densely packed than control leaves (Fig. 4.6A-F). Veins in *AqAS1* treated leaves were much larger than control veins (Fig. 4.6C, F-I) and bulged from the surface of the leaf (Fig. 4.6 D, I-K). Cross sections of one of the radialized lobes (Fig. 4.5I and Fig. 4.6T) revealed that it lacked vasculature entirely (Fig. 4.6L). Marginal regions of the leaf were also much thicker than the controls (Fig 4.6A, D, M-N). SEM images showed that cells on the adaxial surface of the *AqAS1* treated leaves were disorganized and, while smaller on average than comparable cells from control tissue, varied greatly in size (Fig. 4.6O-P, Appendix 4). Near the distal tips of some *AqAS1* treated leaves, the tissue was thicker with multiple ridges on the adaxial side (Fig 4.6Q-R). Increased stomatal density characteristic of the abaxial surface was observed in the patches of tissue between these ridges and on other portions of the adaxial surface (asterisks in Fig. 4.6R-S).

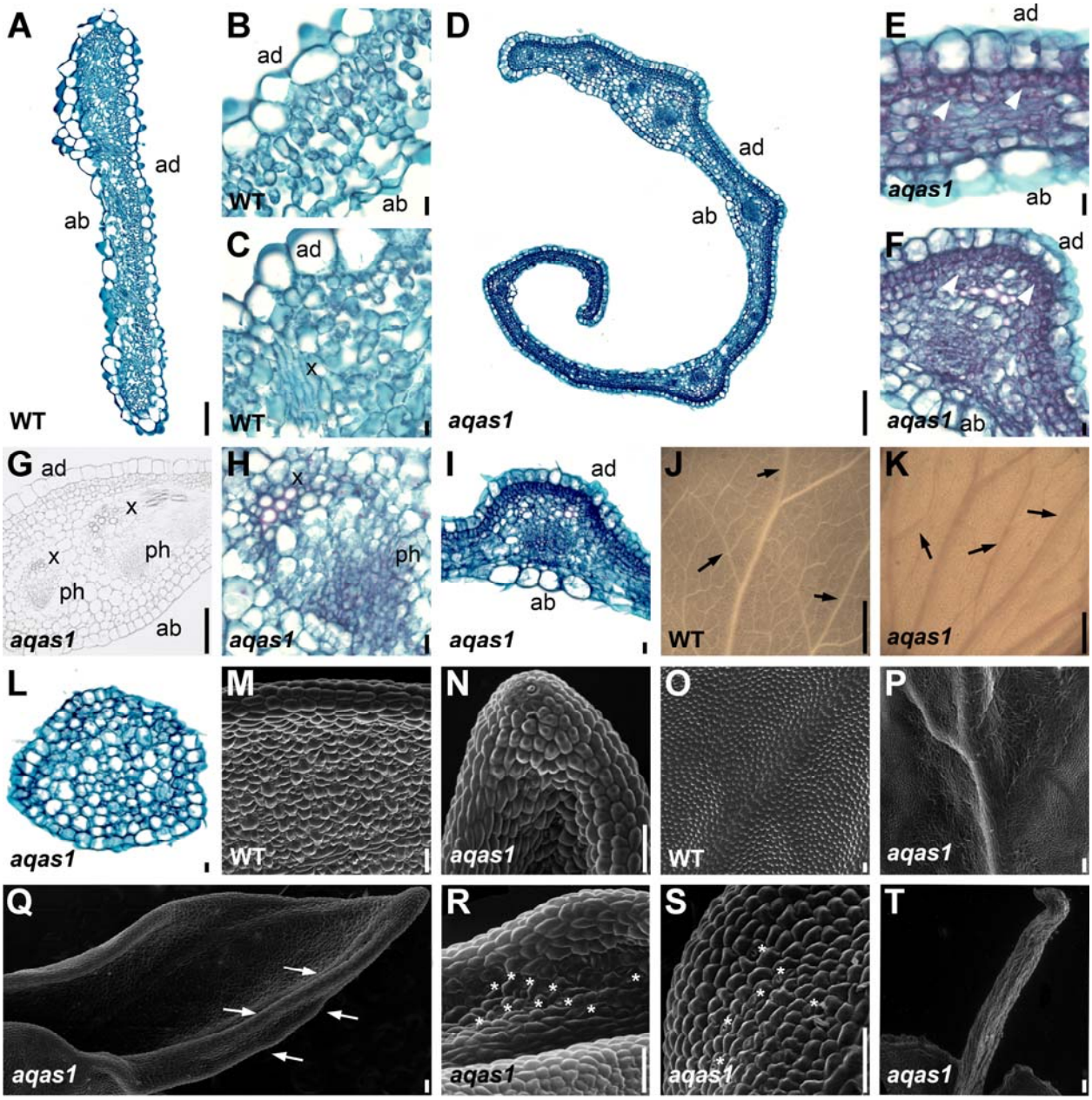


Figure 4.6: Histology of *AqAS1* VIGS treated leaves. **A.** Cross section of an *AqANS* control leaflet (termed throughout as WT). **B.** *AqANS* control leaflet showing internal differentiation of adaxial (ad) and abaxial (ab) surfaces. **C.** A cross section including xylem (x) in an *AqANS* control leaflet. **D.** Cross section of an *AqAS1* VIGS treated leaflet (abbreviated *aqas1* throughout). The leaflet is curled towards the abaxial surface (ab), varies greatly in thickness, and has prominent veins. **E.** *AqAS1* VIGS treated leaflet at higher magnification. Small, densely

Figure 4.6 Continued: packed cells are especially notable in the adaxial (ad) domain (white arrowheads). **F.** A cross section of the vasculature of an *AqAS1* VIGS treated leaf. The veins are more prominent than in the control and the cells over-lying the veins are smaller and more densely packed (white arrowheads). **G.** The margin of an *AqAS1* VIGS treated leaflet, stained for lignin, showing clear polarity of vascular strands (x = xylem, ph = phloem). **H.** An *AqAS1* VIGS treated vein at higher magnification. Polarity of the vein is normal but size is dramatically enlarged relative to C. **I.** Veins in *AqAS1* VIGS treated leaflets protrude above the surface of the leaf. **J.** Cleared *AqANS* control leaflet visualized with dark-field microscopy. Secondary veins are indicated by arrows. **K.** Cleared *AqAS1* VIGS treated leaflet visualized with dark-field microscopy. Secondary veins are larger than those in the control (arrows). **L.** Cross section of radialized lobe of an *AqAS1* VIGS treated leaflet (Fig. 4I). This lobe lacks vasculature. **M-T.** Scanning electron micrographs of *AqANS* and *AqAS1* VIGS treated leaflets. **M.** Margin and abaxial surface of an *AqANS* control leaflet. **N.** Enlarged distal margin of an *AqAS1* VIGS treated leaflet. Curling towards the abaxial surface is evident. **O.** Adaxial surface of an *AqANS* control leaflet. **P.** Adaxial surface of an *AqAS1* VIGS treated leaflet. The cells appear disorganized and vary greatly in size. The veins are much more prominent. **Q.** Multiple ridges (indicated by arrows) along margin of an *AqAS1* VIGS treated leaflet. **R.** Margin at higher magnification. Epidermal tissue between the ridges is characterized by numerous stomata (asterisks), consistent with abaxial identity. **S.** An increased number of stomata (asterisks) are seen on the adaxial surface of the *AqAS1* VIGS treated leaves. **T.** Radial lobe of *AqAS1* VIGS treated leaf. Lateral lobes of leaflet were removed. **A, D, G, and M-T** Scale Bars: 100μm. **B-C, E-F, H-I,** and **L** Scale bars: 10μm. **J and K** Scale Bars: 1mm.

Characterization of *AqAS1* Silenced Plants – Floral Phenotypes

We also observed moderate changes in floral organ morphology in *AqAS1* treated plants (Fig. 4.7). Wild type *Aquilegia* flowers possess five organ types: sepals, petals, stamens, staminodia and carpels (Fig. 4.7A) (Kramer et al. 2007). We have focused on the sepals and petals because they showed stronger phenotypes. Wild type sepals are flat and ovate with an entire margin (Fig. 4.7A-B). The petals are divided into two regions; the proximal spur and the distal limb. Nectar spurs in *Aquilegia coerulea* 'Origami' are typically 5-6 cm in length and slightly curved (Puzey et al. 2012). The limb region is relatively flat with a rounded, weakly lobed margin (Fig. 4.7C). In *AqAS1* treated plants, the margin of the petal limb was very jagged compared to control petals while the petal spurs appeared unaffected (Fig. 4.7D-G). Sepals exhibited similar phenotypes to the leaves in that they were often curled and appeared much narrower than *ANS*-treated sepals (Fig. 4.7D, H-J).

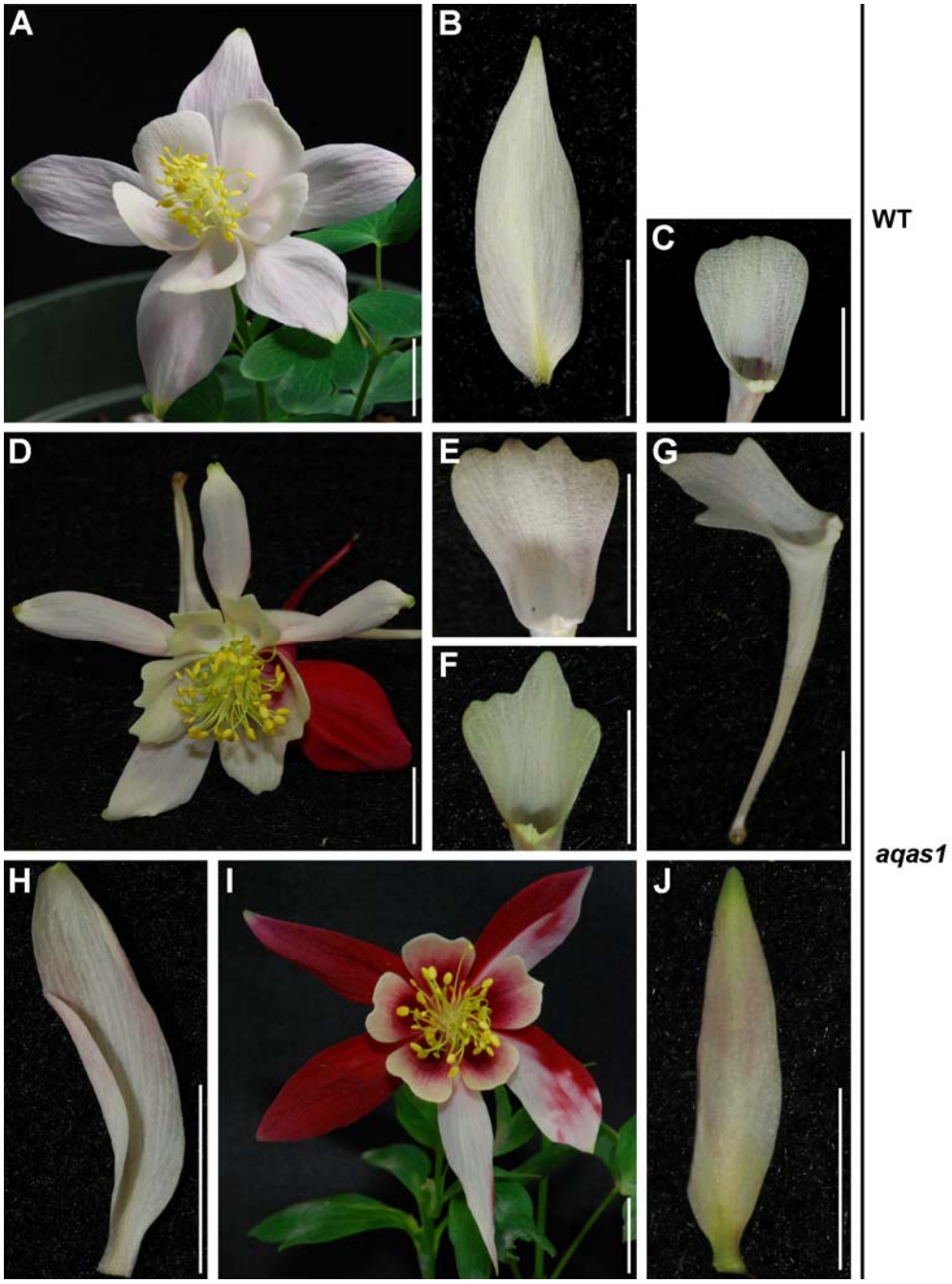


Figure 4.7: *AqAS1* VIGS floral phenotypes. **A-C.** *AqANS*-silenced control flower and perianth organs (WT). **A.** Entire flower. **B.** Sepal. **C.** Petal limb. **D-J.** *AqAS1* VIGS treated flowers and floral organs. **D.** Partially silenced flower. Silenced sepals are narrowed and show weak abaxial curling. Silenced petals have jagged margins. **E-F.** Petal limbs with jagged margins.

Figure 4.7 Continued: G. Silenced petal with jagged margin but normal petal spur. **H.** Curled narrow sepal. **I.** Partially silenced flower. Silenced sepal is narrower than non-silenced sepals. **J.** Narrow sepal. Scale Bars: 1cm.

Assessment of *AqAS1* Down-Regulation

We examined the expression of *AqAS1* in *AqAS1* and *AqANS* control treated tissue using qRT-PCR (Figs. 4.8 and 4.9). Three technical replicates were analyzed for each primer pair on each sample and the data was normalized relative to the expression of the housekeeping gene *AqIPP2*. In the leaves, *AqAS1* expression is variable. Compared to a cDNA pool containing eight *AqANS* treated leaves, three samples appeared to have reduced *AqAS1* expression but one was equivalent and four actually appeared to have increased expression of *AqAS1* (Fig. 4.8A). This pattern may be due to both natural variation in the late expression levels of AqAS1 as well as de-repression of silencing at late stages of development, which is sometimes observed with VIGS (Gould and Kramer 2007; Kramer et al. 2007). We then created two cDNA pools from *AqAS1* treated leaves, one with cDNA from leaves with the highest *AqAS1* expression (AS1 Pool 1) and one containing cDNA from leaves which appeared to have the lowest *AqAS1* expression (AS1 Pool 2). It is notable that the phenotypes of the leaves in these pools were not significantly different. *AqAS1* expression in Pool 2 is ~40% lower than that of the control while expression in Pool 1 is about 2.5 fold higher than the control (Fig. 4.8B).

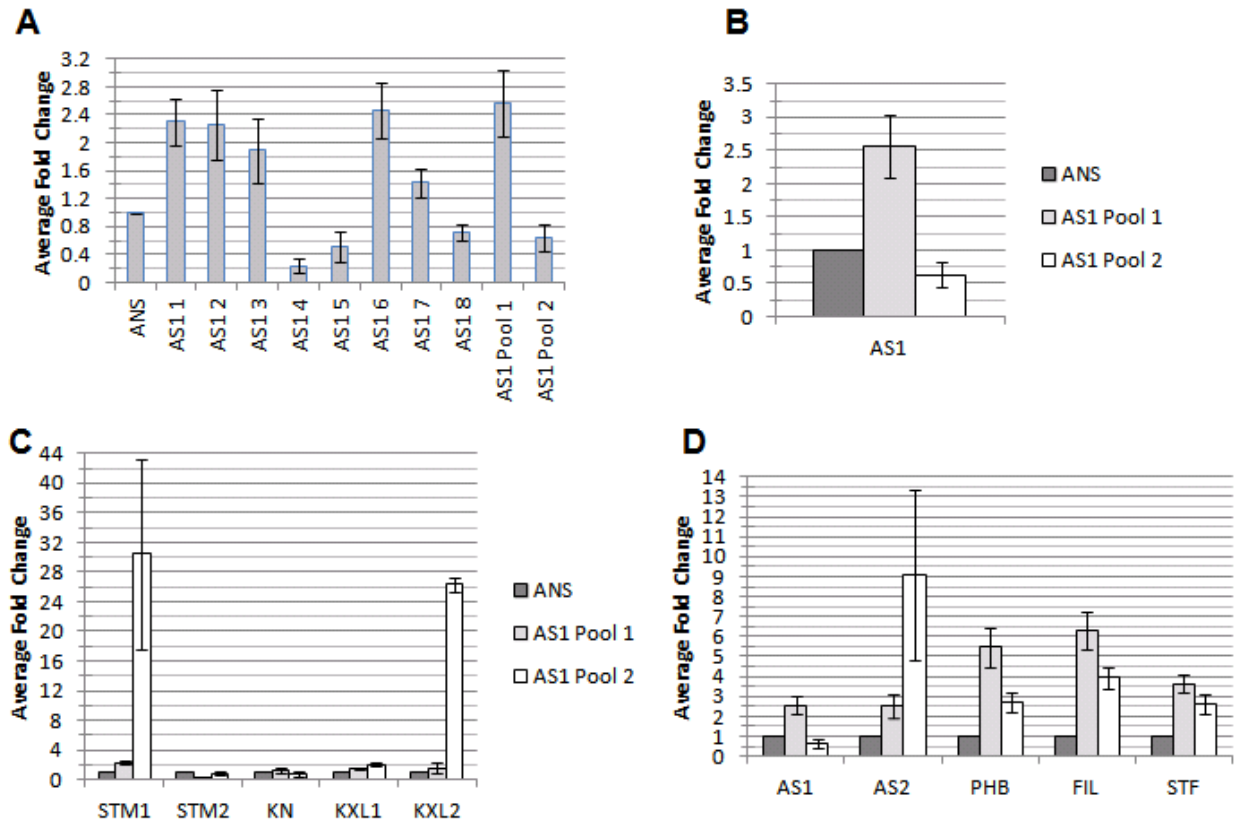


Figure 4.8: qRT PCR analysis of candidate gene expression in *AqAS1* VIGS treated leaves. For each data point, three technical replicates were analyzed. *AqIPP2* expression was used for normalization. **A.** Average fold change in *AqAS1* expression of 8 *AqAS1* VIGS treated leaves and 2 pools of 8 *AqAS1* VIGS treated leaves normalized to a pool of 8 *AqANS* control leaves with SD error bars. **B.** Average fold change in *AqAS1* and class I KNOX gene expression in *AqAS1* VIGS treated leaves normalized to *AqANS* control leaves with SD error bars. cDNA from 8 leaves was pooled to make each sample. **C.** Average fold change in expression of the adaxial identity genes, *AqAS2* and *AqPHB*, the abaxial identity gene *AqFIL*, and the WOX gene, *AqSTF* in *AqAS1* VIGS treated leaves normalized to *AqANS* control leaves with SD error bars. cDNA from 8 leaves was pooled to make each sample.

cDNA from *AqAS1* treated sepals and petals was also divided into two pools for each organ, each containing cDNA from four samples (Fig. 4.9). The pools were created randomly and were not based on phenotype. Compared to a pool of four *AqANS* control sepals, *AqAS1* expression was between 2 and 3 fold higher in the *AqAS1* sepal pools (Fig. 4.9A). *AqAS1* was down-regulated in both petal pools relative to a pool of four *AqANS* control petals, about 50% lower in both cases (Fig. 4.9B).

Assessment of Candidate Gene Expression

Using qRT-PCR we measured the expression of several genes suspected of being downstream targets of *AqAS1* in *AqAS1* and *AqANS* treated tissue, including all five *Aquilegia* class I KNOX genes; the WOX gene associated with laminar proliferation, *STENOFOLIA (AqSTF)*; and several markers of leaf polarity, including the adaxial identity factors *AqAS2* and *AqPHB* and the abaxial identity factor *AqFIL* (Figs. 4.8 and 4.9). In leaves, KNOX gene expression in Pool 1 was similar to that of the control while *AqSTM1* and *KNOX-LIKE2 (AqKXL2)* appeared to be significantly up-regulated in Pool 2 (about 30 fold and 26 fold higher respectively) (Fig. 4.8 C). All of the leaf polarity genes were moderately up-regulated in both leaf pools (Fig. 4.8 D). *AqSTF* up-regulation was also observed in both pools (Fig. 4.8 D). In *AqAS1* treated sepals, several of the KNOX genes were over-expressed compared to the control, particularly *AqSTM1* and *AqKXL2* (Fig. 4.9 A) while *AqSTF* and *AqAS2* were also over-expressed (Fig. 4.9 A). All of the KNOX genes appeared moderately over-expressed in *AqAS1* treated petals, particularly *AqKXL2* (Fig. 4.9 B). *AqSTF* was over-expressed in AS1 Pet Pool 1 while *AqFIL* and *AqAS2* were over-expressed in AS1 Pet Pool 2 (Fig. 4.9 B).

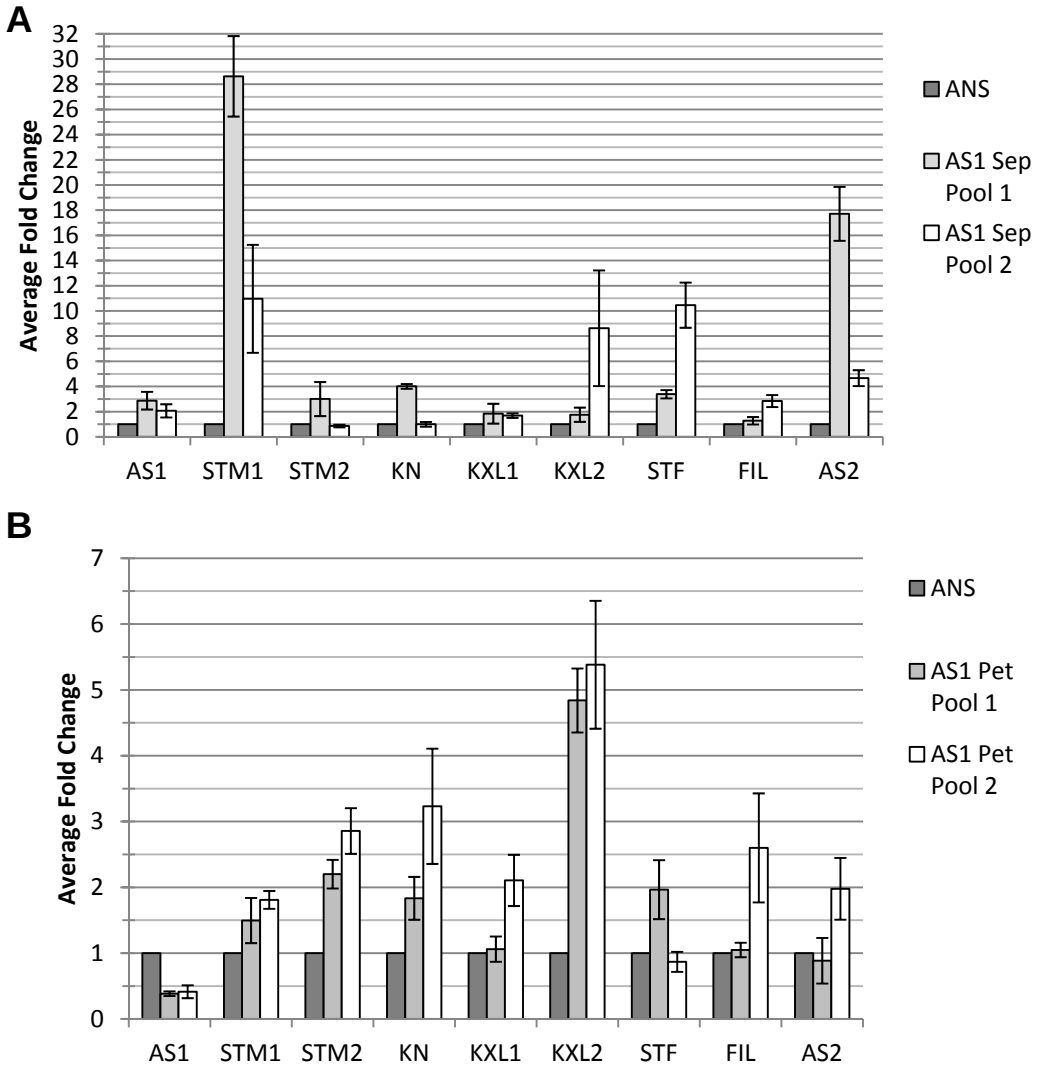


Figure 4.9: qRT PCR analysis of candidate gene expression in *AqAS1* VIGS treated floral organs. cDNA from 4 organs was pooled to make each sample. For each data point, three technical replicates were analyzed. *AqIPP2* expression was used for normalization. **A.** Average fold change in expression of *AqAS1*, the class I KNOX genes, and several leaf polarity genes including *AqAS2,* the abaxial factor *AqFIL,* and the WOX gene *AqSTF* in *AqAS1* VIGS treated sepals normalized to *AqANS* control sepals with SD error bars. **B.** Average fold change in expression of *AqAS1*, the class I KNOX genes, and several leaf polarity genes including *AqAS2,* the abaxial factor *AqFIL,* and the WOX gene *AqSTF* in *AqAS1* VIGS treated petals normalized to *AqANS* control petals with SD error bars.

4.4: Discussion

AS1 and *AS2* have been shown to control KNOX gene regulation, leaf polarity, and laminar expansion in a variety of angiosperms. However, the relative importance of each of these functions and their degree of redundancy with other genetic components appears to vary greatly. In some cases, such as *A. thaliana* and maize, the role of *AS1* in repressing KNOX genes appears to dominate, while in others, like *Antirrhinum*, the function in adaxial identity is more significant (Waites and Hudson 1995; Schneeberger et al. 1998; Byrne et al. 2000). Additionally, *AS1* appears to play a complex role in compound leaf development, both in terms of regulating KNOX gene expression as well as controlling leaflet initiation and outgrowth (Kim et al. 2003a; Kim et al. 2003b). Studies of *AS1* function in compound leaves have been limited to a few species, all of which have pinnately compound leaves (Kim et al. 2003a; Tattersall et al. 2005; Hay and Tsiantis 2006). The impact of down-regulating *AS1* on abaxial-adaxial polarity and branching within the leaf was quite different in each of these species suggesting that, as in species with simple leaves, the relative importance of *AS1*'s functions in compound leaves may also vary by species. Here we sought to further our understanding of the role of *AS1* in compound leaf development by characterizing its expression and function in *Aquilegia,* a model system with palmately compound leaves.

Our analysis started with characterization of the expression of *AqAS1* and that of its presumed binding partner, *AqAS2*. Using qRT-PCR analysis, we found that *AqAS1* and *AqAS2* are expressed throughout *A. coerulea* development with *AqAS1* expression peaking in vegetative tissue while *AqAS2* was expressed at the highest levels in

inflorescence and floral tissue. Lin et al. (2003) measured *AS1* and *AS2* expression throughout *A. thaliana* development and also found that while *AS1* expression drops off in floral organs, *AS2* expression remains high. Thus it is thought that *AS2* may have some *AS1*-independent in floral development (Zhu et al. 2008). *AqAS1* and *AqAS2* expression was further characterized in vegetative meristems with *in situ* hybridization. In species with simple leaves, *AS1* and the KNOX genes are expressed in non-overlapping domains with KNOX gene expression being confined to the meristem and *AS1* expressed only in developing leaves, while in many species with compound leaves both *AS1* and the KNOX genes are expressed together in the SAM and in leaf primordia (Waites et al. 1998; Tsiantis et al. 1999; Byrne et al. 2000; Kim et al. 2003a). However, in *Cardamine* and pea, *AS1* expression is not seen in the SAM, and in pea the class I KNOX genes are not expressed in leaf primordia (Tattersall et al. 2005; Hay and Tsiantis 2006). In *Aquilegia*, *AqAS1* and *AqAS2* are expressed in developing leaf primordia and the SAM, and their expression domain overlaps with that of the class I KNOX genes, similar to the pattern seen in a number of compound leafed species by (Kim et al. 2003a). In both simple and compound leaf primordia, *AS1* or *AS2* can be asymmetrically localized (ex. *A. thaliana AS2* and tomato *AS1*), expressed throughout the primordia (ex. *A. thaliana AS1* and *Antirrhinum PHAN*), or restricted to the medial zone between the abaxial and adaxial domains (ex. Pea, *Cardamine*, and *Nicotiana*) (Waites et al. 1998; Byrne et al. 2000; Iwakawa et al. 2002; Kim et al. 2003a; McHale and Koning 2004; Tattersall et al. 2005; Hay and Tsiantis 2006). Early in *Aquilegia* leaf development *AqAS1* and *AqAS2* expression is seen throughout the primordia, but at

least in the case of *AqAS1* it may become restricted to the medial zone later in development.

Next, we used VIGS to down-regulate *AqAS1.* VIGS treated leaves were deeply lobed and curled toward the abaxial surface with enlarged veins that protruded from the surface of the leaf. The adaxial mesophyll cells of the VIGS treated leaves were often smaller than wild type cells and much more densely packed. In some leaves, leaflets were arranged around the entire circumference of the terminus of the petiole or rachis. These phenotypes are most similar to *as1* mutants in simple leafed species where the phenotypes appear to be primarily caused by ectopic KNOX gene expression. Mutations in *as1* and *as2* in *A. thaliana* result in downward curling leaves (Byrne et al. 2000), while vascular abnormalities, especially enlargement of the secondary vasculature, have been reported in *as1* mutants in many species (Waites and Hudson 1995; Byrne et al. 2000; Sun et al. 2002; McHale and Koning 2004; Tattersall et al. 2005). Enlarged secondary vasculature as well as increased lobing in the leaves and small undifferentiated cells are also phenotypes observed in KNOX gene over-expressing lines (Smith et al. 1992; Chuck et al. 1996). Indeed, we find that in two *Aquilegia* KNOX genes, *AqSTM1* and *AqKXL2,* are significantly over-expressed in leaves with reduced *AqAS1* expression. This ectopic KNOX expression could explain most of the phenotypes we observed. For example, *AqSTM1* and *A1KXL2* could aberrantly promote pluripotency and cell divisions in the adaxial compartment of the leaf, thus, explaining the histology of the VIGS treated leaf cells, Furthermore, the resultant difference in cell number between the top and bottom portions of the lamina

could cause the leaves to curl. Thus, we believe that, as in several other simple- and comound-leafed species, *AqAS1* appears to play a major role in regulating the class I KNOX genes in *Aquilegia.*

However, what is quite interesting is that this over-expression of *AqSTM1* and *AqKXL2* does not appear to have an effect on branching within the leaf or leaflet number, as has been seen in other species with compound leaves and as would be predicted by the general model for KNOX-dependent compound leaf development (Bharathan et al. 2002; Kim et al. 2003a; Hay and Tsiantis 2006). Additionally, we observed only a weak effect on leaflet placement, again contrary to what would be predicted based on previous observations (Kim et al. 2003a). This may be due to incomplete down-regulation of *AqAS1* in our VIGS treated plants, however, it is also important to note that loss of *AS1* expression does not always have the same effect on compound leaf development. In tomato, leaves with reduced *LePHAN* expression are less compound and often, in the most severe cases, completely radialized. This is thought to be because both *LePHAN* and KNOX gene expression are required for leaflet initiation in tomato (Kim et al. 2003a; Kim et al. 2003b). In *Cardamine*, however, *ChAS1* RNAi lines have more highly branched leaves (Hay and Tsiantis 2006). Mutations in the pea *AS1* homolog, *crispa (cri),* cause leaves to become more complex and *PsKN2*, the pea *BP* homolog is ectopically expressed in the leaves, but the arrangement of the leaflets is not affected and the leaves remain pinnate (Tattersall et al. 2005). Unlike in other species with compound leaves, however, pea KNOX genes are never expressed in leaf primordia and branching is believed to be controlled by *UNIFOLIATA,* the pea *LEAFY*

homolog (Hofer et al. 1997). Thus, there is likewise reason to believe that, as in pea, KNOX genes may not be sufficient to promote leaflet branching in *Aquilegia*. Along these lines, we have previously used VIGS to target members of a general negative epigenetic regulatory complex, the Polycomb Repressive Complex 2 (PRC2) in *Aquilegia* (Gleason and Kramer in prep). In these experiments, we often observed an increase in complexity in PRC2 down-regulated leaves, but we could not detect any ectopic KNOX gene expression in these leaves. Taken together, these results appear to suggest that class I KNOX expression is neither sufficient nor required for increased branching in the compound leaves of *Aquilegia*. Further evidence for possible novel pathways functioning in *Aquilegia* leaf complexity comes from a study by (Pabón-Mora et al. 2013), who observed a decrease in leaf complexity when they down-regulated *AqFL1,* a MADS box gene normally involved in floral meristem identity. All of these data suggest that the control of leaf complexity in *Aquilegia* may not follow standard models, as has independently observed in the derived legumes.

The *AqAS1* VIGS treated leaves also had some evidence of aberrant abaxial-adaxial patterning. One of these leaves had needle-like lobes on all of the leaflets but, unfortunately, its lack of vasculature and extremely simple morphology made it impossible to determine whether they were truly radialized. More convincingly, we often observed patches of abaxial tissue along the adaxial margin of some leaves, which were often surrounded by ectopic ridges. Similar laminar outgrowths have been seen in other *as1* mutants, including *Antirrhinum* and pea, and are consistent with the hypothesis that the juxtaposition of abaxial and adaxial identity is required for laminar

outgrowth (Waites and Hudson 1995; Tattersall et al. 2005). Possibly in relation to these outgrowths, the *Aquilegia* WOX gene, *AqSTF,* is moderately (2-4 fold) up-regulated in our *AqAS1* VIGS treated leaves. In a number of models, members of the WOX family have been implicated in laminar expansion as factors that act to maintain cell division in the “marginal meristem” (Vandenbussche et al. 2009; Tadege et al. 2011b; Nakata et al. 2012; Lin et al. 2013). The observed *AqSTF* over-expression could, therefore, be due to the ectopic outgrowths or possibly because of regulatory interactions between *AqAS1* and the WOX genes. During laminar expansion in *A. thaliana*, the WOX genes *PRS* and *WOX1* are thought to negatively regulate *AS2* expression, as well as the expression of many other adaxial and abaxial identity genes, but it is unclear if *AS1* and *AS2* themselves feedback onto WOX gene expression as well (Nakata et al. 2012). This WOX gene over-expression, together with that of the class I KNOX, could also be contributing to the over-proliferation of mesophyll cells in the adaxial compartment of the *AqAS1*-silenced leaves.

As another sign of disruption in proper leaf polarity, in *AqAS1* VIGS treated leaves we saw increases in several genes controlling abaxial and adaxial identity, including the adaxial identity factors *AqAS2* and *AqPHB* as well as the abaxial identity gene *AqFIL.* There are two possible explanations for these observations. First, mis-expression of *AqSTF* could be affecting *AqAS2*, *AqPHB* and *AqFIL* expression as feedback between the adaxial/abaxial and medial pathways has been suggested (Tadege, Personal Communication). Alternately the increased number of cells, particularly on the adaxial surface could result in increased *AqPHB* and *AqAS2* expression while the minor

abaxialization of the leaves could lead to an increase in *AqFIL* expression. Further characterization of the regulatory interactions between these genes in *Aquilegia* is necessary in order to fully assess these findings.

AqAS1 also appears to play a role in sepal and petal development in *Aquilegia. In situ* hybridization showed that both *AqAS1* and *AqAS2* are expressed at the margins of developing floral organs. *AqAS1* VIGS treated sepals were narrower than the controls and often curled towards the adaxial surface while the petals were largely unaffected, except for observed jagged margins in the petal limb. Interestingly, the effects of *AqAS1* down-regulation on KNOX gene expression was much broader in floral organs: all five *Aquilegia* class I KNOX genes were ectopically expressed, especially *AqSTM1* in sepals and *AqKXL2* in sepals and petals. The regulatory interactions between the KNOX genes and *AS1* are likely to be different in these lateral organs than in the compound leaves since floral organs are not compound and almost no KNOX gene expression has been observed in *Aquilegia* floral tissue (Collani et al. in prep). Given the significant KNOX over-expression, it is somewhat surprising that the petal phenotypes we observed were relatively minor. The deep lobing at the margin of the petal is very consistent with expected KNOX over-expression phenotypes (Chuck et al. 1996), but the class I KNOX genes have also been suggested to play a role in spur development and we observed no effect on spurs in our *AqAS1* VIGS treated flowers (Golz et al. 2002; Box et al. 2011). This finding is consistent with (Collani et al. in prep), who found no evidence for

class I KNOX gene expression in developing petals and is, therefore, additional evidence that the loci are not playing a role in *Aquilegia* spur development.

4.5: Conclusions

- Similar to what has been observed in other species with compound leaves, *AqAS1* and *AqAS2* are expressed in both the SAM and leaf primordia, and their expression in developing leaves overlaps with that of the class I KNOX genes.
- *AqAS1* acts to repress several of the class I KNOX genes in developing leaves and contributes to adaxial identity and laminar expansion, possibly by regulating the WOX gene *AqSTF*.
- However, despite ectopic KNOX gene in *AqAS1* VIGS treated leaves, no increase in branching was observed. This, along with evidence from other studies, suggests that the class I KNOX genes may be neither necessary nor sufficient to promote branching in the compound leaves of *Aquilegia.*
- The effects of *AqAS1* down-regulation on KNOX gene expression was much broader in floral organs, however, petal spur development was not affected in these plants. Thus, KNOX gene over-expression does not perturb petal spur development in *Aquilegia*, unlike what has been observed in other models.
- While *AS1* homologs appear to control KNOX gene expression and adaxial identity in many angiosperms, the relative importance of these roles and their overall affect on leaf morphology varies greatly by species.

Chapter 5:

General Conclusions and Discussion

In this study we sought to further our understanding of gene regulation during lateral organ development in the lower eudicot *Aquilegia.* Gene expression can be controlled at the level of transcription by several mechanisms, including via transcription factors and epigenetic regulation. Therefore, we examined the evolution, expression, and function of both a deeply conserved epigenetic regulatory complex, Polycomb Repressive Complex 2 (PRC2), and a transcription factor with important functions in leaf development, *ASYMMETRIC LEAVES 1* (*AS1*). Chapters 2 and 3 focused on PRC2, a complex that epigenetically represses gene expression a number of multicellular organisms across the animal and plant kingdoms. In the plant model system, *Arabidopsis thaliana*, PRC2 has many important developmental functions including promoting proper endosperm development, regulating the transition to flowering in response to vernalization, and restricting the expression of several transcription factors with important roles in lateral organ development. While some studies in other model systems, particularly in the grasses, suggests that some of these PRC2 functions may be conserved, relatively little is known about the PRC2 outside of the major angiosperm models. In Chapter 4, our focus shifts to the control of leaf development by the R2-R3 type MYB, *AS1*. Studies in several model systems bearing either simple or pinnately compound leaves have revealed complex roles for *AS1* homologs in the establishment of leaf polarity and the regulation of varying degrees of indeterminacy, as controlled by the class I KNOX genes. The current study is the first to examine homologs of *AS1* outside the major core eudicot and grass models or in a system bearing palmately compound leaves.

Our study of PRC2 homologs began with the identification of homologs of the *Aquilegia* PRC2 loci and VEL PHD family. We found that, unlike many plant model systems, *Aquilegia* has a simple PRC2 complex with no recent duplications. In *A. thaliana* and barley, PRC2 is thought to be important in the vernalization response. Therefore, we next examined the expression of the PRC2 loci and several VEL PHD genes in the vernalization responsive species *Aquilegia vulgaris.* The PRC2 genes are broadly expressed throughout *A. vulgaris* development with no obvious tissue or stage specialization. Furthermore, VEL PHD gene expression in *A. vulgaris* is not confined to vernalization as seen with *VIN3* in *A. thaliana,* but moderately increases both during vernalization and in the inflorescence. Next, we addressed the role of *Aquilegia* PRC2 in endosperm development by testing if the ancient paralogs *AqCLF* and *AqSWN* are imprinted in the endosperm. Unlike PRC2 loci in maize, rice, and *Arabidopsis*, *AqCLF* and *AqSWN* do not appear to be imprinted in *Aquilegia* endosperm.

The next step was to examine the function of the PRC2 complex in lateral organ development using a reverse genetic tool, Virus-Induced Gene Silencing (VIGS). Plants that treated with *AqFIE* or *AqEMF2* VIGS constructs had a range of phenotypes. Most notably, the leaves often had ruffled or curled lamina, additional lobing, and an increased frequency of higher order branching. The sepals were narrow and curled while the petals were narrow, stunted or had bent spurs. The petal limbs also had a particularly intense yellow coloration due to an accumulation of carotenoid pigments in these cells. This role for the PRC2 in carotenoid regulation has not been observed in other species. However, we found that, as in *A. thaliana*, floral ABC class MADS box

gene family members, *AG*, *SEP3*, and possibly *AP3*, were targets of the *Aquilegia* PRC2, suggesting that a role in maintaining the repression of these genes in lateral organs may be conserved across eudicots. However, another family targeted by the *A. thaliana* PRC2, the class I KNOX genes, were not ectopically expressed in PRC2 down-regulated tissue in *Aquilegia.* Given the increase in higher order branching we observed in PRC2 down-regulated leaves, this was particularly surprising because the KNOX genes have been hypothesized to promote leaf complexity across many species with compound leaves.

Having discovered this novel aspect of compound leaf development - that branching was increased without ectopic KNOX expression - we sought to understand the role of the transcription factor *AqAS1* in lateral organ development. *AS1* appears to regulate compound leaf development both by restricting the expression of the class I KNOX genes and by promoting adaxial identity, laminar expansion, and leaflet arrangement. However, *AS1* function has not been studied in species with palmately compound leaves. We first studied the expression of *AqAS1* and its binding partner *ASYMMETRIC LEAVES 2* (*AqAS2*) in *A. coerulea* using both qRT-PCR and *in situ* hybridization. Similar to what has been observed in other species with compound leaves, *AqAS1* and *AqAS2* are expressed in both the SAM and leaf primordia, and their expression in developing leaves overlaps with that of the class I KNOX genes. Next, we examined the function of *AqAS1* in lateral organ development using VIGS. *AqAS1* VIGS treated leaves were deeply lobed and curled toward the abaxial surface with enlarged veins that protruded from the surface of the leaf. No affect on leaf complexity was observed.

Sepals were narrower than the controls and often curled towards the adaxial surface, while the petals were largely unaffected, except for observed jagged margins in the petal limb. Several of the class I KNOX genes were ectopically expressed in *AS1* VIGS treated tissue, with a larger number of homologs being affected in the floral organs. While the class I KNOX genes have been suggested to affect petal spur development in other species, KNOX gene over-expression does not perturb petal spur development in *Aquilegia.* However, despite ectopic KNOX gene in *AqAS1* VIGS treated leaves, no increase in branching was observed.

Thus, we have two consistent, independent sets of data. Leaf branching increased in *AqFIE* and *AqEMF2* VIGS treated tissue, but the class I KNOX genes were not ectopically expressed in these leaves. In *AqAS1* VIGS treated leaves, the KNOX genes were over-expressed, but no effect on leaf branching was observed. Taken together these data suggest that the class I KNOX genes may be neither necessary nor sufficient to promote branching in the compound leaves of *Aquilegia.* Class I KNOX genes are thought to establish indeterminacy in leaf primordia and allow for leaflet initiation in many diverse taxa with complex leaves (Hareven et al. 1996; Bharathan et al. 2002; Hay and Tsiantis 2006). However, in at least one species, pea, leaf complexity is controlled by *UNIFOLIATA*, a homolog of the floral meristem identity gene *LEAFY* (Hofer et al. 1997). Like pea, *Aquilegia* may have independently evolved a novel mechanism of controlling leaf complexity.

However, the methods we have used to study the role of the class I KNOX genes in leaf complexity so far are indirect. They affect genes that may control KNOX gene transcription rather than directly targeting the KNOX genes themselves. Unfortunately, multiple attempts to knock-down one or more class I KNOX genes using VIGS were unsuccessful. Of course, one major drawback to VIGS silencing is that it is transient and can be lost at any point during development. Since VIGS does not affect the entire plant and we cannot identify silenced tissue until the organ has matured, it is impossible to examine candidate gene expression at early developmental stages. Therefore it is possible that the KNOX genes were over-expressed early on in PRC2 VIGS treated tissue but that they were later silenced by other factors. Future studies should seek to more directly assess the role of the class I KNOX genes in *Aquilegia* lateral organ development. An ideal method to do this would be through the use of transgenics. Members of the Kramer lab are working to develop a protocol for producing transgenic *Aquilegia,* which will allow constitutive knock-down of one or more class I KNOX genes with artificial miRNAs, as well as localized over-expression. These types of experiments will allow the role of class I KNOX genes in both leave and petal development to be fully explored.

Future studies should also focus on identifying novel genes that may be acting to promote leaflet branching in *Aquilegia.* One approach to do this would be to combine transgenic plants containing *AqFIE* RNAi constructs with high throughput sequencing technique (RNAseq). Transgenic leaf tissue with increased branching due to reduced *AqFIE* expression could be collected and sequenced to look for genes that are up- or

down-regulated relative to wild type tissue. This list of candidate genes could then be further reduced by identifying genes that are expressed in leaf primordia around the time of leaflet initiation and by testing the function of these genes with VIGS or through further transgenics. In addition, such *AqFIE*-silenced plants could be used to more broadly explore the target repertoire of the PRC2 complex in *Aquilegia*. Comparisons of RNAseq data from silenced and wildtype leaves as well as floral organs would undoubtedly reveal a large number of differentially expressed genes that are normally suppressed by PRC2, including the critical players in the carotenoid production pathway. Further application of ChIPseq targeting histone modifications would narrow this initial list to just the genes that are likely to be direct targets, and potentially identify other critical loci, such as those controlling vernalization response in *Aquilegia*. Of course, another attractive option would be to use forward mutagenesis, but this will require over-coming the serious inbreeding depression problem displayed by *A. x coerulea* 'Origami'

Overall, these studies have demonstrated that *Aquilegia* is likely to be a useful model for exploring the conservation of PRC2 targeting and *AS1* homolog function, as well as identifying novel features in both of these pathways. This highlights the importance of exploring many different angiosperm lineages in order to test major hypotheses regarding patterns of conservation and convergence.

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Appendices

Appendix 1: Table of Primers

	Gene	Forward Primer	Reverse Primer
VIGS:	*AqANS*	GGTCTAGATTGGGATTGGAAGAAGAAAGGC	AAGGATCCATGTTGAGCAAATGTGCGA
	AqAS1	CGGAATTCCGGAGGGTGGAACTACAACTTGAGTCG	GCTCTAGAGCTGGAGGACCAAACAAATTCA
	AqCLF	CGGAATTCCGTGATTGGCAGTGATGACACA	CGTCTAGACGTTGGAATGCCCTTCAACAG
	AqEMF2	CGGAATTCCGTTGTTGGCATCACATGACCT	GCTCTAGAGCCATCAGGAGAGCCCAGTTTC
	AqFIE	CGGAATTCCGATTTGCTGGAGCGTATGGAC	GCTCTAGAGCCAAGCCACCTATTGCAGTCA
	AqSWN	CGGAATTCCGTCGAAGCTTCTAGGGTTGCT	GCTCTAGAGCTGGTGGCGGTACTTCTTGAT
Sequencing:	*AqAS2*	GATCATTCAAGTCTCATTGCAGCCAT	
		CATGAATGTGTCTACAAACATTGGAC	
	AqCLF	ATAGTTGGCCGAAGGAGGAT	
		CAGGTTGAGATTTATTCGTTGGAG	
		GGATCAACCATGTCATCAATATAAC	
		CTTCATCCTTTCTCGC	
	AqEMF2	GCTGCTGAAGAAAGTCTTTCAGTCTACTGC	GCTTGCAATCTATACTCTCACTGTCGTAACCG
		GTGTGGAAGCTTTAAGGCCTTAGG	GCAGGAGTGTGCGGTTTCTGG
	AqFIE	CCAAGTTCTTCTACCCTAAACCTTCCTACCC	
		CCAAAAATGGCGAAAACAACCTTAGG	GATTCCCTTCGGCATTACAA
	AqMSI1	CACTTCGTTTGCCCTATTTCCCTCC	CTCAGTTCCCTCTCTCTGGCCTC
	AqSWN	TCGAAGCTTCTAGGGTTGCT	
		ATCGGAGAATGCCTGATGAT	
	AqVIN3A	GGAGGAAAAGAGAGAACTTGTTTATGAG	CATGCAGAACCCACTTGACACAGC
			GCTCCAAGCTTCCTCACAGCCTC
	AqVIN3B	CCGAACTTAGCGTGCAGAGC	CCATCATCTACATCACCACTTGTCG
	AqVRN5	TCAGAAATCCCAGTCGGTGT	CGGCTCTACACGCAGAATTT
		TCCTGATTCCGCTGAAGAGT	AACCAGGCCTGTGAATCATC
			CCCAAGCAATACGCAAAATC
5' RACE:	*AqCLF*		CGGTAGGCATCAAGAACAAACTGGTC
			CACCGTCGCCGCAACTTATCCG
			CCAAGGGTAGTGAACTGAGGACAAGG
			GGACGGACCTCCAA
			CCTTGTCCTCAGTTCACTACCCTTGG
	AqEMF2		CCAACAAGTGATACCTAAG
			GTCCATTCCTTCACCATCTTGAGC
			GTTCTGCACCTGCACCCC
	AqFIE		GACTGGAGTTCCCAAAC
			GCCTTCCCTATTACCTATTGCTGCTGTTTTG
			CCCCTCCTTTGGCTTTGGTTCCC
			AAGTCCACCAGCAACC
			CCTAAGGTTGTTTTCGCCATTTTTGG
			GGATTCCCTTCGGCATTACAAGC
	AqVIN3B		GCTGAAGATACCAAACTG
			GCCTGTTCACAATACCCCGTGCC
			CCACTCGCCTCGTGTCCTTTGC
			CCACTCGCCTCGTGTCCTTTGC
Imprinting Primers:	*AqCLF*	TTGCCAAGGAACGGATATGT	AGAGTCTGGCTCATTACATGC
		CAGATCATGCTCCTGCTTGG	AAGGCCAATGATAAGACAACAAA
	AqSWN	TTGGTTGTGGAGATGGTTCA	TCCGCTGTATTAACTATTAAGAATGG

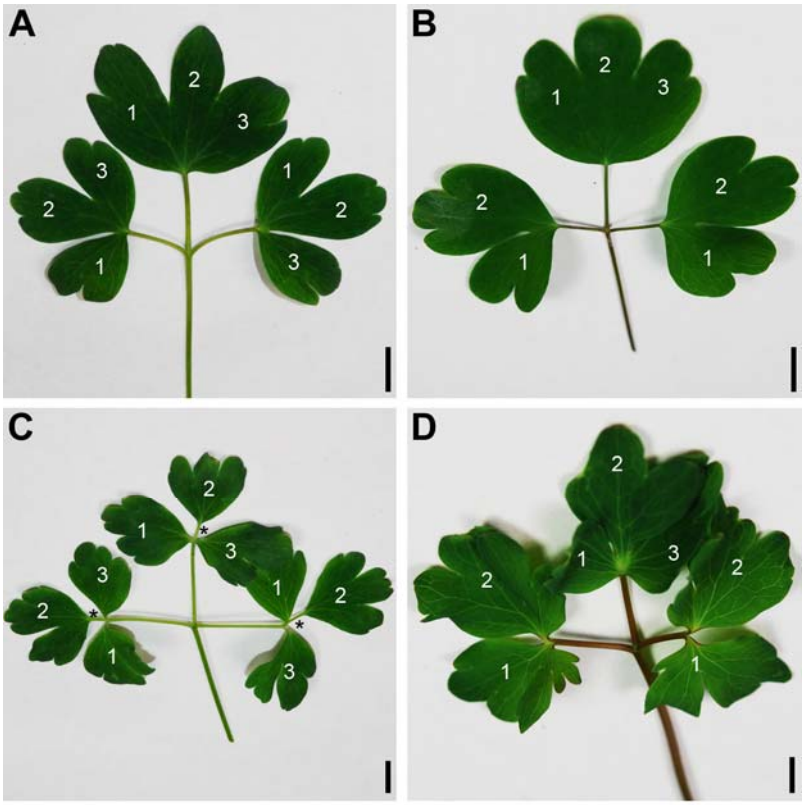
Appendix 1 Continued: Table of Primers

	Gene	Forward Primer	Reverse Primer
in situ:	*AqAS1*	TCACCTTCTACAGTCACTCCTCC	CATTTGGTTCAATAAGCCTTGG
	AqAS2	CAATGGTGGTGCAGCTTATG	CTGTCTCATATTCACCAGACGA
	AqHIS4	AAGGCGTGGTGGTGTTAAGCGTATCA	GAATTACAAGAAAGTAGTAGATCAGAATCCAAC
	AqKN	TCAAGGAGGAGAAAGGATTGG	CTCGTGGATCAATTTCAGGAAC
	AqSTM1	ATTATCCAAGGCTCTTAGCTTG	CCGGTCAAAAGCATCACCAC
	AqSTM2	CCTCACTATTCTCGTCTCTTAGCTGC	TTAGATCCGCAACCTCATGATATCC
RT and qRT PCR:	*AqAG1*	GGCTTGCACAGACTCTACCA	CGCAGTTTTGTCACTTCTTGC
	AqAG2	ACTTTAACTGTGTCAGAAGCGAATTTG	ACTCTCCCACCAATTTCCTGTTATG
	AqANS	CCATGCCACCAACTTCCTTTTCTAGCC	CATGTCAACCTGGCCTAAGACACCTACTG
	AqAP3-1	GAGAGTTCTGTAAAGATTGTTGCA	AAGCACCATTAGCCGCCATTT
	AqAP3-2	TGAGAGAATGCAAGATACCCTGC	CTTCAAAGTCAAGTGCTGGAAAGC
	AqAP3-3	TCTACCACAACGAAGAAGATATACGA	CCCGAACAAGTTTCACAGACTCATC
	AqAS1	GGACATCGAGCTTGGGTAGC	CCTGCTAATTGCTCTCTATACTCTGC
	AqAS1	GTATTGCAGAAGAAGAGCTGTGTTG	CATGCGCTGAGACACAAGATTC
	AqAS2	CCAGAACGTAGGCCTCACTGGAC	CCCGGGAGATTAAGAATTGATGG
	AqCCD4	CATCTCCTCCTCCCAAATCA	AGGAAGTAGGGGGTGCTTGT
	AqCCD4L	GCATTCCAGTACAGCCCAAT	TTTTGGTGATGGCAAAATCA
	AqCLF	ACTCTTGCGGTTATTGATTGC	TGCCAATCAACTTTCGCTTA
	AqCRTISO	ATTCCTGGTGGAAGTTCTGG	CCTCCATCTCACACCCAACT
	AqEMF2	CTCATGGGAAAAGTGTGCAA	ATGCAGCTTTCCTTTTCCAA
	AqFIE	TCCACTGAAACCTTCCCTTG	TCGTAGGAAATTTTGATGGAAGA
	AqFIE	ACTTTTGGTTGCTGGTGGAC	CAAGGGAAGGTTTCAGTGGA
	AqFIL	GCGTGGATTGCTTCTTCCTA	AATGACCGGCATCATGAGAT
	AqFL1	GGAAATATAAGCACCCCTCATCA	CCAAATTACAATCAAAGCAACAACTG
	AqIPP2	CAGGTGAAGACGGACTGAAGTTATC	CCAAGACTGGAAAAAAGACCACAC
	AqKN	GGCAATGGAGTTTATGAGAAGG	TTGATCCTCAGACGAACC
	AqKXL1	AAAGTGGATCTGAGATGATGAGCGCGATCTG	TGGCCTACTTTCTCGGCTAA
	AqKXL2	CAAGGAAGTAGTGAAGGAAGTGGTGATATG	CGAGTTGAAACGGCACCTCT
	AqMSI1	GTGGTGAGATTGGGGGTTTT	ATATAGCGTGCCCGATTGAC
	AqNCED3	TCCATACCAAGTTCGCATCA	TGATCAGGGATCACCACAAA
	AqPHB	AACAATTGACAAATCCAGCTGCTGTG	AGTGGAGTTGAGTTTTGCTGTTGTTGG
	AqPSYL1	CGGCCTTACGACATGTATGA	GCTCATCAAAGCTTTTGTATCTTG
	AqPSYL2	ATCTCTTCCAAGGTCGTCCA	CGACTTCTTCAGGTCCAACC
	AqSEP1	TGCGAGAAGCTAACAAAACG	CCTGCTTCCCATAGGAGTTG
	AqSEP2	ATATTGCCGTCAACCTGCTC	AGCTGGTGCTGCAACTGTAA
	AqSEP3	GAGGACCAGAGCCCAATGTA	TCCACTCAATGGTCCCAAAT
	AqSTF	CCAAACACTGGGGTTTCACT	CATGGCATGTCTATCAACCAA
	AqSTM1	CCAAAAGAAGCTAGGCAGCA	TCCTGTCGATTCTGCAAGTG
	AqSTM2	CCTCACTATTCTCGTCTCTTAGCTGC	GCGTAGGCCTCTTCCAACTT
	AqSTM2	CCTCACTATTCTCGTCTCTTAGCTGC	TTAGATCCGCAACCTCATGATATCC
	AqSWN	TCGAAGCTTCTAGGGTTGCT	TGACCCATTCTGATCCACTG
	AqVIN3A	GGCCTTCGATATCAGCTTTG	TGGCTGAGAAAGAGACGATG
	AqVIN3B	CTGTGGGAAAGTGAATGACC	AGTACATCCACTCGCCTCGT
	AqVRN5	TCAGAAATCCCAGTCGGTGT	ATCCTTTTGGTCTGGGGTGT
	AqWUS	GGGATATGGATCTGTTGTTATGGAGA	GCTCGACACCCGCACCAA

Appendix 2: Gene Identification Numbers

My Gene Name	Phytozome Locus Name	Genbank Accession Number
AqANS	Aquca_013_00483	DQ229152
AqAG1	Aquca_136_00009	AY464111
AqAG2	Aquca_022_00039	AY464110
AqAP3-1	Aquca_006_00074	EF489478
AqAP3-2	Aquca_006_00072	EF489477
AqAP3-3	Aquca_007_00336	EF489476
AqAS1	Aquca_027_00369	**Submitted to Genbank**
AqAS2	Aquca_058_00006	**Submitted to Genbank**
AqCCD4	Aquca_053_00008	---
AqCCD4L	Aquca_053_00007	---
AqCLF	Aquca_015_00009	JN944600
AqCRTISO	Aquca_004_00557	---
AqEMF2	Aquca_053_00026	JN944598
AqFIE	Aquca_015_00396	JN944599
AqFIL	Aquca_013_00693	---
AqFL1	Aquca_002_00915	DT758909
AqHIS4	Aquca_027_00434	DT44843
AqIPP2	Aquca_058_00173	**Submitted to Genbank**
AqKN	Aquca_133_00021	**Submitted to Genbank**
AqKXL1	Aquca_003_00549	---
AqKXL2	Aquca_002_00080	---
AqMSI1	Aquca_026_00353	JN944602
AqNCED3	Aquca_125_00036	---
AqPHB	Aquca_026_00358	---
AqPSYL1	Aquca_026_00362	---
AqPSYL2	Aquca_091_00082	---
AqSEP1	Aquca_006_00411	JX680244
AqSEP2	Aquca_002_00916	JX680245
AqSEP3	Aquca_011_00121	JX680247
AqSTF	Aquca_091_00003	---
AqSTM1	Aquca_012_00046	**Submitted to Genbank**
AqSTM2	Aquca_032_00057	**Submitted to Genbank**
AqSWN	Aquca_003_00541	JN944601
AqVIN3A	Aquca_081_00015	JN944603
AqVIN3B	Aquca_096_00026	JN944604
AqVRN5	Aquca_001_00585	JN944605
AqWUS	Aquca_004_00664	---

Appendix 3



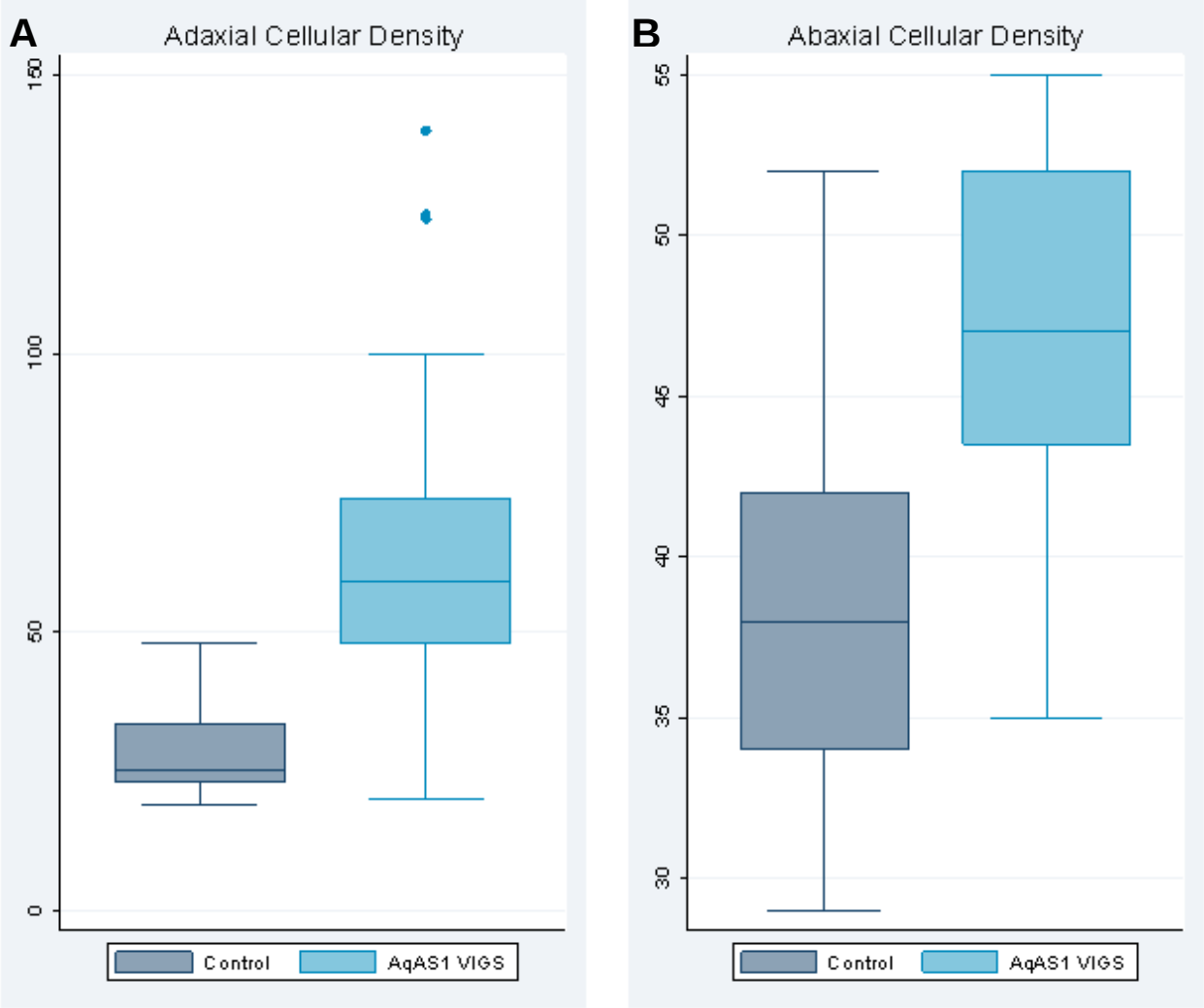
E

	Petiolules per Lateral Unit	Petiolules per Medial Unit
WT Unvernalized	0	0
AqFIE Unvernalized	0.5 ± 0.8	0.1 ± 0.4
WT Vernalized	0.6 ± 0.5	0.6 ± 0.5
AqFIE Vernalized	1.15 ± 1.6	0.4 ± 0.5

Heteroblasty in *Aquilegia coerulea*: A-D. Leaf forms in wild type *A. coerulea.* Leaf lobes are numbered. **A.** Unvernalized leaf with 3 major lobes per leaflets. **B.** Unvernalized leaf with 2 major lobes in the lateral leaflets. **C.** Vernalized leaf with higher order petiolules where the central lobe of each leaflet is a separate leaflet borne on a petiolule (asterisks). **D.** Vernalized leaf with 2 major lobes in the lateral leaflets. These leaves are more deeply lobed than similar unvernalized leaves. **E.** Average petiolules per medial and lateral leaflets with standard deviations.

Heteroblasty in *Aquilegia coerulea* Continued: Both unvernalized and vernalized *AqFIE* silenced lateral leaflets had on averaged more petiolules than the wild type. Unvernalized *AqFIE* silenced lateral leaflets also had a slightly higher average number of petiolules compared to wild type, but vernalized *AqFIE* silenced leaves had a slightly lower number of petiolules per medial leaflet. When quantified, this increase is significant (*) at $p<0.05$ for unvernalized lateral leaflets but not significant for the other stages/leaflet types.

Appendix 4



Cellular Density in *AqAS1* VIGS Treated Leaves: The number of cells in 40,000μm^2 area. **A.** Adaxial surface. Cells of AqAS1-treated leaves are on average significantly smaller, though there is a high degree of variability in cell size. There were three significant outliers in the data set. **B.** Abaxial surface. On average, cells of *AqAS1* leaves are slightly smaller than those of the controls.

Appendix 5: List of Abbreviations

AG – AGAMOUS

Am – Antirrhinum majus

ANS – ANTHOCYANIN SYNTHASE

AP – APETALA

Aq – Aquilegia

AqC – Aquilegia canadensis

AqV – Aquilegia vulgaris

AS1 – ASYMMETRIC LEAVES 1

AS2 – ASYMMETRIC LEAVES 2

At – Arabidopsis thaliana

BLAST – Basic Local Alignment Search Tool

Bn – Brassica napus

bp – base pair(s)

BP – BREVIPEDICELLUS

CAF1 – Chromatin Assembly Factor 1

CCD – CAROTENOID CLEAVAGE DIOXYGENASE

cDNA – DNA complementary to RNA

Ch – Cardamine hirsuta

ChIP – Chromatin Immuno-Precipitation

CLF – CURLY LEAF

CRI – CRISPA

CRTISO – CAROTENOID ISOMERASE

Cs – Citrus sinensis

DFCI – Dana-Farber Cancer Institute

DNA – Deoxyribonucleic Acid

DNase – deoxyribonuclease

E(z) – Enhancer of Zeste

Ec – Eschscholzia californica

Ed – Endosperm

Em – Embryo

EMF – EMBRYONIC FLOWER

ESC – Extra Sex Combs

EST – Expressed Sequence Tag

EtOH – Ethanol

Eudicots – eudicotyledonous

FAA – Formaldehyde Acetic Acid Alcohol

FIE – FERTILIZATION INDEPENDENT ENDOSPERM

FIL – FILAMENTOUS FLOWER

FIS – FERTILIZATION INDEPENDENT SEED

FL1 – FRUITFUL-Like 1

FLC – FLOWERING LOCUS C

FT – FLOWERING LOCUS T

Gm – Glycine max

H3K27 – Histone H3 Lysine 27

H3K27me3 – Histone H3 Lysine 27 trimethylation

H3K4 – Histone H3 Lysine 4

Hd3a – Heading date 3 a

HIRA – Histone Cell Cycle Regulation Defective Homolog

HOX – Homeobox

Hv – Hordeum vulgare

IG1 – INDETERMINATE GAMETOPHYTE 1

Inflo – Inflorescence

IPP2 – Isopentyl Pyrophosphate:Dimethylallyl Pyrophosphate Isomerase

JLO – JAGGED LATERAL ORGANS

KAN – KANADI

KN – KNOTTED

KNAT – KNOTTED-LIKE FROM ARABIDOPSIS THALIANA

KNOX – *knotted1* homeobox gene

KXL – KNOX-LIKE

Le – Solanum lycopersicum

Lj – Lotus japonicas

LOB – Lateral Organ Boundaries

MADS – *MCM1, AGAMOUS, DEFICIENS, SRF*

Mbp – Mega base pair

Md – Malus x domestica

MEA – MEDEA

Mez1 – Maize E(z)1

Migu – *Mimulus guttatus*

MKN – MOSS KNOTTED1-LIKE

ML – Maximum Likelihood

MSI – MULTI COPY SUPPRESSOR OF IRA

Mt – *Medicago truncatula*

MYB – Myeloblastosis

n – chromosome number

NCED – 9-CIS-EPOXYCAROTENOID DIOXYGENASE

Ns – Nicotiana sylvestris

Nt – Nicotiana tabacum

oligo – oligodeoxyribonucleotide

Os – Oryza sativa

Pavi – Panicum virgatum

PcG – Polycomb Group

PCR – Polymerase Chain Reaction

Pet – Petal

PHAN – PHANTASTICA

PHB – PHABULOSA

PHE1 – PHERES 1

Phv – Phaseolus vulgaris

PHV – PHAVOLUTA

Pin – *Pinus*

Pp – Physcomitrella patens

Ppl – Populus

PRC1 – Polycomb Repressive Complex 1

PRC2 – Polycomb Repressive Complex 2

Ps – Pisum sativum

PSY – PHYTOENE SYNTHASE

qRT-PCR – quantitative Real Time Polymerase Chain Reaction

Que – Quercus

RACE – Rapid Amplification of cDNA Ends

RAxML – Randomized Axelerated Maximum Likelihood

REF6 – RELATIVE OF EARLY FLOWERING 6

REV – REVOLUTA

RNA – Ribonucleic Acid

Rs – Raphanus sativus

RS2 – Rough Sheath 2

RT-PCR – Reverse Transcriptase PCR

SAM – Shoot Apical Meristem

SEM – Scanning Electron Microscopy

Sep – Sepal

SEP – SEPALLATA

SNP – Single Nucleotide Polymorphism

SOC1 – SUPPRESSOR OF CONSTANS 1

St – Solanum tuberosum

STF – STENOFOLIA

STM – SHOOTMERISTEMLESS

Su(z)12 – *Suppr*essor of Zeste12

SWN – *SWINGER*

Ta – *Triticum aestivum*

TCP – Teosinte Branched 1-Cycloidea-PCF

TRV – Tobacco Rattle Virus

UTR – Unstranslated region(s)

VEL – *VERNALIZATION5/VIN3-LIKE PROTEIN*

VEL PHD – Vernalization 5/VIN3-Like Plant Homeodomain

VIGS – Virus-Induced Gene Silencing

VIN3 – *VERNALIZATION INSENSITIVE3*

VRN – *VERNALIZATION*

Vu – *Vigna unguiculata*

Vv – *Vitus vinifera*

WOX – Wuschel Related homeobox

YAB3 – *YABBY3*

Zm – *Zea mays*